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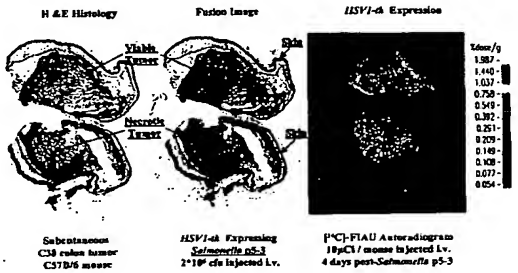

(57) Abstract: The present invention provides non-invasive compositions and methods to detect solid tumors using tumor-targeted bacteria. specifically, the present invention provides compositions and methods for detecting solid tumors *in vivo* by delivery of a marker gene to a solid tumor via a population of tumor-targeted bacteria, methods for detecting solid tumors by detecting a compound incorporated into the bacteria, methods for detecting solid tumors by detecting an infection caused by the bacteria at the tumor site(s), methods for detecting solid tumors by detecting an antigen present on the surface of the bacterial vectors. When detecting a tumor entails detecting a marker gene product expressed by the tumor-targeted bacteria, the marker gene product can be detected directly in the tumor, by the use of a labeled moiety that interacts with the marker gene product, or by the use of the labeled marker substrate. Thus, if a marker gene product is directly detectable, it can be detected directly. When a marker gene product is not directly detectable, a labeled moiety that interacts with the marker gene product is detected, or a labeled marker substrate is used and a labeled marker metabolite is detected. A tumor can thus be localized or detected by scanning a subject to detect the marker gene product, a labeled complex comprising the marker gene product and its interacting moiety, or a labeled marker metabolite, respectively, thereby imaging the tumor. Specifically, the attenuated tumor-targeted bacteria of the invention are facultative aerobes or facultative anaerobes which are modified to encode the marker gene. Still further, this invention provides compositions and methods of simultaneously imaging and treating a tumor in a subject using one or a plurality of populations tumor-targeted organisms. The invention provides non-invasive, clinically applicable methods for imaging tumors which can be implemented using existing imaging techniques to monitor and evaluate *in vivo* cancer treatments in human subjects. Kits are also provided.

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NON-INVASIVE TUMOR IMAGING BY TUMOR-TARGETED BACTERIA

This application claims priority to U.S. provisional patent application No. 60/157,620, filed on October 4, 1999, the content of which is incorporated herein by reference its entirety.

1. Field of the Invention

The present invention is concerned with non-invasive imaging methods to detect solid tumors *in vivo*. The invention is also concerned with non-invasive methods to monitor and/or treat solid tumors.

2. Background of the Invention

2.1. Cancer Chemotherapy

A major problem in the chemotherapy of solid tumor cancers is delivery of therapeutic agents, such as drugs, in sufficient concentrations to eradicate tumor cells while at the same time minimizing damage to normal cells. Thus, studies in many laboratories are directed toward the design of biological delivery systems, such as antibodies, cytokines, and viruses for targeted delivery of drugs, pro-drug converting enzymes, and/or genes into tumor cells. *See e.g.*, Crystal, 1995, Science 270: 404-410.

2.2. Bacterial Infections and Cancer

Early clinical observations reported cases in which certain cancers were reported to regress in patients with bacterial infections, See Nauts *et al.*, 1953, Acta Medica Scandinavica 145:1-102, (Suppl. 276); Shear, 1950; J.A.M.A. 142:383-390. Since these observations, Lee *et al.*, 1992, Proc. Natl. Acad. Sci. USA 89:1847-1851 (Lee *et al.*) and Jones *et al.*, 1992, Infect. Immun. 60:2475-2480 (Jones *et al.*) isolated mutants of *Salmonella typhimurium* that were able to invade HEp-2 (human epidermoid carcinoma) cells *in vitro* in significantly greater numbers than the wild type strain. The "hyperinvasive" mutants were isolated under conditions of aerobic growth of the bacteria that normally repress the ability of wild type strains to invade HEp-2 animal cells. However, such hyperinvasive *Salmonella typhimurium* as described by Lee *et al.* and Jones *et al.* carry the risk of pan-invasive infection and could lead to wide-spread bacterial infection in the cancer patient.

Carswell *et al.*, 1975, Proc. Natl. Acad. Sci. USA 72:3666-3669, demonstrated that mice injected with bacillus Calmette-Guerin (BCG) have increased serum

levels of TNF and that TNF-positive serum caused necrosis of the sarcoma Meth A and other transplanted tumors in mice. As a result of such observations, immunization of cancer patients with BCG injections is currently utilized in some cancer therapy protocols. See Sosnowski, 1994, Compr. Ther. 20:695-701; Barth and Morton, 1995, Cancer 75 (Suppl. 2):726-734; Friberg, 1993, Med. Oncol. Tumor. Pharmacother. 10:31-36 for reviews of BCG therapy.

However, TNFα-mediated septic shock is among the primary concerns associated with bacteria, and can have toxic or lethal consequences for the host (Bone, 1992, JAMA 268: 3452-3455; Dinarello *et al.*, 1993, JAMA 269: 1829-1835). Further, dose-limiting, systemic toxicity of TNFα has been the major barrier to effective clinical use. Modifications which reduce this form of an immune response would be useful because TNFα levels would not be toxic, and a more effective concentration and/or duration of the therapeutic vector could be used.

Further, resistance to antibiotics can complicate eliminating the presence of bacteria within the human body (Tschape, 1996, D T W Dtsch Tierarztl Wochenschr 1996 103:273-7; Ramos *et al.*, 1996, Enferm Infec. Microbiol. Clin. 14: 345-51).

2.3. Tumor-targeted Bacteria

Genetically engineered *Salmonella* have been demonstrated to be capable of being tumor-targeted, possess anti-tumor activity and are useful in delivering genes such as the herpes simplex virus thymidine kinase (HSV TK) to solid tumors (Pawelek *et al.*, WO 96/40238).

Pawelek *et al.* (1997, Cancer Res. 57:4537-4544) employed a gene fusion consisting of the β-lactamase secretion signal followed by the entire coding sequence of HSV1-tk. The gene product of this construct was secreted into the periplasmic space of the bacterium and was shown to be functionally active. Antitumor studies with a strain carrying this construct demonstrated that treatment with ganciclovir resulted in an enhancement of antitumor activity only when the HSV1-tk plasmid was present, thus establishing the therapeutic potential of *Salmonella* expressing functionally active HSV1-tk.

2.4. Decreased Induction of TNFα by Modified Bacterial Lipid A

Modifications to the lipid composition of tumor-targeted bacteria which alter the immune response as a result of decreased induction of TNFα production were suggested by Pawelek *et al.* (Pawelek *et al.*, WO 96/40238). Pawelek *et al.* provided methods for isolation of genes from *Rhodobacter* responsible for monophosphoryl lipid A (MLA) production. MLA acts as an antagonist to septic shock. Pawelek *et al.* also suggested the

use of genetic modifications in the lipid A biosynthetic pathway, including the mutation *firA*, which codes for the third enzyme UDP-3-O (R-30 hydroxylmyristoly)-glucosamine N-acyltransferase in lipid A biosynthesis (Kelley *et al.*, 1993, J. Biol. Chem. 268: 19866-19874). Pawelek *et al.* showed that mutations in the *firA* gene induce lower levels of TNFα. In *Escherichia coli*, the gene *msbB* (*mlt*) which is responsible for the terminal myristylation of lipid A has been identified (Engel, *et al.*, 1992, J. Bacteriol. 174:6394-6403; Karow and Georgopoulos 1992, J. Bacteriol. 174: 702-710; Somerville *et al.*, 1996, J. Clin. Invest. 97: 359-365). Genetic disruption of this gene results in a stable non-conditional mutation which lowers TNFα induction (Somerville *et al.*, 1996, J. Clin. Invest. 97: 359-365; Somerville, WO 97/25061).

Hone and Powell, WO97/18837 ("Hone and Powell"), disclose methods to produce gram-negative bacteria having non-pyrogenic Lipid A or LPS. Hone and Powell propose using non-pyrogenic bacteria only for vaccine purposes.

Maskell, WO98/33923, describes a mutant strain of *Salmonella* having a mutation in the *msbB* gene which induces TNFα at a lower level as compared to a wild type strain.

Bermudes *et al.*, WO 99/13053, teach compositions and methods for the genetic disruption of the *msbB* gene in tumor-targeted *Salmonella*, which result in *Salmonella* possessing a lesser ability to elicit TNFα and reduced virulence compared to the wild type. In certain embodiments, some such mutant *Salmonella* have increased sensitivity to chelating agents as compared to wild type *Salmonella*. In other embodiments, the mutant tumor-targeted *Salmonella* deliver a gene product such as a pro-drug converting enzyme useful as an anti-tumor agent.

2.5. Imaging Gene Therapy

Blasberg and Tjuvajev developed a non-invasive imaging system to detect gene transfer and expression in target tissues which is useful for monitoring and evaluating *in vivo* gene therapy (Tjuvajev *et al.*, 1995, Cancer Res. 55:6126-6132; Tjuvajev *et al.*, 1996, Cancer Res. 56:4087-4095; Tjuvajev *et al.*, 1998, Cancer Res. 58:4333-4341. The system uses a transfer vector containing HSV1-TK and a labeled marker substrate to non-invasively image target tissue or cells which have taken up the marker gene.

Imaging methods have been described for monitoring gene therapy in United States Patent No. 5,703,056 entitled "Non-Invasive Imaging of Gene Transfer" issued December 30, 1997 to Blasberg and Tjuvajev. Although a variety of vectors are used to image gene therapy, these references do not discuss the use of tumor-targeted, facultative aerobic or facultative anaerobic bacteria or bacterial vectors, alone or in combination with

other agents, to detect tumors by imaging or to treat solid tumors. Further, these references do not discuss the use of facultative aerobic or facultative anaerobic bacterial vectors for delivery of any substance therapeutic to solid tumor sites.

Citation or identification of any reference in Section 2, or any section of this application shall not be construed as an admission that such reference is available as prior art to the present invention.

3. Summary of the Invention

The present invention provides non-invasive methods to detect solid tumors *in vivo* by delivery of a marker gene to a solid tumor. The vector for delivery of the marker gene to the solid tumor is a heterogeneous or homogenous population of tumor-targeted bacteria. The present invention also provides other non-invasive methods to detect solid tumors using tumor targeted bacterial vectors. Such methods comprise, *in vivo*, detecting a compound incorporated into the tumor-targeted bacterial vectors, detecting an infection caused by the bacterial vectors, or detecting an antigen present on the surface of the bacterial vectors after the bacterial vectors have been administered to a subject.

When a marker gene is delivered to a solid tumor, the marker gene can be detected directly in the tumor, by the use of a labeled moiety that interacts with the marker gene product, or by the use of a labeled marker substrate. More particularly, in one embodiment, the invention encompasses use of attenuated tumor-targeted bacteria or bacterial vectors, such as, *e.g.*, *Salmonella,* as a vector for the delivery of a marker gene to an appropriate site of action, *e.g.*, the site of a solid tumor. If the marker gene product itself is detectable by non-invasive methods, the marker gene product itself can be detected directly. Alternatively, a labeled moiety that interacts with the marker gene product is detected, or a labeled marker substrate is used and a labeled marker metabolite is detected. A tumor can thus be localized or detected by scanning a subject to detect the marker gene product, a labeled complex comprising the marker gene product and its interacting moiety, or a labeled marker metabolite, respectively, thereby imaging the tumor. Specifically, the attenuated tumor-targeted bacteria of the invention are facultative aerobes or facultative anaerobes which are modified to encode the marker gene.

In one embodiment, the marker gene encodes a fluorescent protein and the marker gene product is detected directly. In another embodiment, the marker gene encodes strepatividin and is detected by administering labeled biotin, in which case the marker gene is indirectly detected by detection of the labeled biotin. In another embodiment, the marker gene product is a heterologous protein that is immunologically detectable, and the marker gene product is detected by administration of a labeled antibody.

In a specific embodiment, the marker gene is HSV1-tk or VZV-tk and the marker substrate is a labeled 2'-fluoro-nucleoside analogue. In a preferred embodiment, the labeled marker substrate is a labeled 2'-fluoro-5-iodo-1-beta-D-arabinofuranosyl-uracil (FIAU). In other specific embodiments, the labeled marker substrate is a radiolabeled acyclovir (ACV) or a radiolabeled ganciclovir (GCV). As disclosed herein, the 2'-fluoro-nucleoside analogues were chosen for use in the subject invention because they are selectively phosphorylated by HSV1-TK or VZV-TK, can be radiolabeled with appropriate radionuclides for imaging *e.g.*, with SPECT or PET and are resistant to metabolic degradation *in vivo* (see, for example, Abrams *et al.*, 1985, Int. J. Appl. Radiat. Isot. 36: 233-238) thereby facilitating interpretation of the resultant images and measurements.

In other embodiments of the invention, the tumor-imaging methods of the invention do not require the use of a marker gene. In certain specific embodiments, tumor imaging comprises administering a labeled compound to the subject, which labeled compound is preferentially incorporated into the bacteria, and detecting the labeled compound. In other specific embodiments, the tumor is imaged by scanning to image an infection caused by the bacteria at tumor site.

In addition to initial tumor detection, the present invention also provides a non-invasive method to monitor the course of disease or effectiveness of tumor treatment. Repeated imaging of tumor tissue during the course of treatment allows direct observation of changes in size. In addition to detection and monitoring, the present invention also provides methods for therapy to inhibit tumor growth or reduce the tumor size. HSV1-TK and VZV-TK metabolize marker substrates as well as pro-drug substrates, and may be genetically engineered to prefer a specific substrate.

The present invention is based, in part, on the surprising discovery that the metabolic activities of tumor-targeted bacteria are of sufficient magnitude and duration while in tumor cells or the tumor environment to be able to express, incorporate, trap, bind or otherwise hold within the tumor region, sufficient amounts of a detectably labeled agent to allow non-invasive detection of the tumor. For example, expression of a β-lactamase:HSV1-TK fusion protein with a periplasmic localization signal has been found to cause the trapping effect of phosphorylation of a marker substrate (*e.g.*, FIAU) and permits signal localization for monitoring. Accordingly, according to the present invention, in another embodiment, the marker gene product of a tumor-targeted bacterial vector is retained in the cytosol and periplasm of the vector, where such vector is used for imaging or monitoring a tumor. In another embodiment, the marker gene product of a tumor-targeted bacterial vector is retained in the cytosol of the vector, where such vector is used for imaging or monitoring a tumor.

The present method employing genetically engineered tumor-targeted bacteria, such as *Salmonella*, can be used to detect solid tumors which are at least 2 times, more preferably 4-5 times, and most preferably 10 times smaller than can be detected using current methods including but not limited to x-ray and CT scanning. Advantageously, the method can be used to detect and/or monitor tumor metastases as well as primary solid tumors. Unlike viral vectors which can only accommodate a small segment of exogenous DNA, bacteria, such as *Salmonella*, can accept multiple genes either by insertion into the bacterial chromosome or by transformation with multiple plasmids.

This invention provides a method of detecting a tumor by imaging in a subject comprising: (a) administering to the subject a tumor-targeted bacteria containing a marker gene, wherein the tumor-targeted bacteria targets the tumor cells and/or the tumor environment and the marker gene is expressed in the tumor-targeted bacteria, thereby generating a marker gene product; and (b) scanning the subject to detect the marker gene product, thereby imaging the tumor in the subject. In certain embodiments of the invention, the marker gene is detected directly. In other embodiment, the marker gene is detected indirectly. In one embodiment, indirect detection further comprises administering to the subject a labeled marker-binding moiety after step (a), wherein the marker gene product of step (a) binds to the labeled marker-binding moiety; and wherein said scanning is after clearance of residual labeled marker-binding moiety not bound to the marker gene product, thereby detecting the labeled marker-binding moiety localized to the tumor and imaging the tumor in the subject. In another embodiment, indirect detection further comprises administering to the subject a labeled marker substrate after step (a), wherein the tumor-targeted bacteria expressing the marker gene product of step (a) metabolizes the labeled marker substrate to produce a labeled marker metabolite; and wherein said scanning is after clearance of residual marker substrate not metabolized by the marker gene product, thereby detecting the labeled marker metabolite localized to the tumor and imaging the tumor in the subject.

This invention provides a method of detecting a tumor by imaging in a subject comprising: (a) administering to the subject a tumor-targeted bacteria, wherein the tumor-targeted bacteria targets the tumor cells and/or the tumor environment; (b) administering to the subject a labeled compound that is preferentially incorporated into the tumor-targeted bacteria; and (c) scanning the subject to detect the labeled compound, thereby detecting the tumor-targeted bacteria and imaging the tumor in the subject. In a preferred embodiment, the tumor-targeted bacteria is a mutant having an enhanced preference to incorporate the labeled compound. In a preferred mode of the embodiment, the bacteria is a mutant at the *asd* locus and the marker compound is diaminopimelic acid.

The present invention further provides methods of imaging a tumor in a subject comprising (a) administering to the subject a tumor-targeted bacteria, wherein the tumor-targeted bacteria targets the tumor cells and/or the tumor environment, and (b) scanning to image an infection caused by the bacteria, thereby detecting and imaging the tumor in the subject. In a preferred embodiment, imaging the infection comprises detecting sequestered polymorphonuclear neutrophils at the site of infection, for example by using a labeled antibody that detects an antigen, such as CD15, present on the polymorphonuclear neutrophils, or by using a labeled chemotactic peptide analog that binds to a receptor present on the polymorphonuclear neutrophils.

The present invention provides methods of imaging a tumor in a subject comprising: (a) administering to the subject a tumor-targeted bacteria, wherein the tumor-targeted bacteria targets the tumor cells and/or the tumor; (b) administering a labeled antibody to the subject, wherein the antibody binds to an antigen present on the surface of the tumor-targeted bacteria; and (c) scanning the subject to detect the labeled antibody, thereby imaging the tumor in the subject. In a preferred embodiment, the antigen present on the surface of the tumor-targeted bacteria is an O-antigen, an H-antigen, or an outer membrane protein.

Further, the present invention provides a method of monitoring a tumor during the course of a disease and/or treatment in a subject comprising: (a) administering to the subject a tumor-targeted microorganism containing a marker gene, wherein the tumor-targeted microorganism targets the tumor cells and/or the tumor environment and the marker gene is expressed in the tumor-targeted microorganism, thereby generating a marker gene product; (b) scanning the subject to detect the marker gene product, thereby imaging the tumor in the subject; and (c) repeating steps (a) and (b) as needed during the course of the disease in the subject.

Further, the present invention provides a method of monitoring a tumor during the course of a disease and/or treatment in a subject comprising: (a) administering to the subject a tumor-targeted bacteria, wherein the tumor-targeted bacteria targets the tumor cells and/or the tumor environment; (b) administering to the subject a labeled compound that is preferentially incorporated into the tumor-targeted bacteria;(c) scanning the subject to detect the labeled compound, thereby detecting the tumor-targeted bacteria and imaging the tumor in the subject; and (d) repeating steps (a) through (c) as needed during the course of the disease in the subject.

Further, the present invention provides a method of monitoring a tumor during the course of a disease in a subject comprising (a) administering to the subject a tumor-targeted bacteria, wherein the tumor-targeted bacteria targets the tumor cells and/or

the tumor environment; (b) scanning to image an infection caused by the bacteria, thereby detecting and imaging the tumor in the subject; and (c) repeating steps (a) and (b) as needed during the course of the disease in the subject.

Yet further, the present invention provides a method of monitoring a tumor during the course of a disease in a subject comprising (a) administering to the subject a tumor-targeted bacteria, wherein the tumor-targeted bacteria targets the tumor cells and/or the tumor; (b) administering a labeled antibody to the subject, wherein the antibody binds to an antigen present on the surface of the tumor-targeted bacteria; (c) scanning the subject to detect the labeled antibody, thereby imaging the tumor in the subject; and (d) repeating steps (a) through (c) as needed during the course of the disease in the subject.

Still further, this invention provides methods of simultaneously imaging and treating a tumor in a subject. In one embodiment, simultaneous imaging and treatment comprises administering tumor-targeted bacteria expressing a marker gene and a suicide gene capable of converting a prodrug into a cytotoxic drug, together with a labeled marker substrate. A single tumor-targeted bacterial vector may contain more than one bacterial expression construct, wherein at least one construct is used for imaging the tumor, as above, and at least one construct is used for treating the tumor. Alternatively, a population of at least two tumor-targeted bacterial vectors is administered, wherein one vector is for imaging as above, and another vector is for therapeutic treatment.

The invention provides a non-invasive, clinically applicable method for imaging tumors which can be implemented using existing imaging techniques to monitor and evaluate *in vivo* cancer treatments in human subjects.

Yet further, the invention provides compositions for carrying out the non-invasive imaging (and optionally treatment) methods of the invention, said compositions comprising a population of the tumor-targeted bacteria of the invention suitable for non-invasive tumor imaging. In one embodiment, a composition of the invention comprises a population of tumor-targeted bacteria which harbor a recombinant streptavidin gene operably linked to a promoter. In a preferred mode of the embodiment, the promoter is preferentially active at the tumor site or in the environment. In another embodiment, the tumor-targeted bacteria is a partial *asd* mutant with an enhanced preference to incorporate labeled DAP.

Yet further, the invention provides pharmaceutical compositions comprising a population of the tumor-targeted bacteria of the invention suitable for non-invasive tumor imaging, and a pharmaceutically acceptable carrier.

Yet further, the invention provides kits comprising compositions of the invention for carrying out the non-invasive detection methods of the invention, as well as

for carrying out the non-invasive detection and treatment methods of the invention. Specifically, the invention provides kits comprising in one or more containers (a) a purified population of tumor-targeted bacteria and (b) a detectably labeled molecule. In certain embodiments, the bacteria contain a marker gene operably linked to a promoter. In one embodiment, the labeled molecule is a labeled moiety which binds to the marker gene product. In a preferred mode of the embodiment, the labeled molecule is labeled biotin and the marker gene is streptavidin. In another embodiment, the labeled molecule is a labeled substrate of the marker gene product. In other embodiments, the labeled molecule is a labeled compound which is preferentially incorporated into the tumor-targeted bacteria. In one embodiment, the tumor-targeted bacteria is a mutant having an enhanced preference to incorporate the labeled compound. In one mode of the embodiment, the bacteria is mutant at the *asd* locus and the labeled compound is DAP. In yet other embodiments, the labeled molecule is a labeled antibody that detects an antigen present on polymorphonuclear neutrophils or on the surface of the tumor-targeted bacteria, or a labeled chemotactic peptide analog that binds to a receptor present on polymorphonuclear neutrophils.

Facultative aerobic and facultative anaerobic bacteria useful for the methods and compositions of the present invention include but are not limited to *Escherichia coli* (including but not limited to pathogenic (*e.g.*, entero-invasive or uropathogenic) *Escherichia coli)*, *Salmonella spp.*, *Shigella spp.*, *Streptococcus spp.*, *Yersinia enterocolitica*, *Listeria monocytogenies*, and *Mycoplasma hominis.* In a preferred embodiment, the bacterial vector is an attenuated strain of *Salmonella* genetically engineered to express an altered Lipid A which reduces the virulence of the strain, and genetically engineered to express a gene product which aids in preventing the growth of a solid tumor, which gene product is under the control of an irradiation-inducible promoter.

Illustrative examples of solid tumors include, but are not limited to, sarcomas, carcinomas, lymphomas and other solid tumor cancers, such as renal carcinoma, mesoendothelioma, bladder cancer, germ line tumors and tumors of the central nervous system, breast cancer, prostate cancer, cervical cancer, uterine cancer, lung cancer, ovarian cancer, testicular cancer, thyroid cancer, astrocytoma, glioma, pancreatic cancer, stomach cancer, liver cancer, colon cancer, and melanoma.

3.1. Definitions

As used herein, *Salmonella spp.* encompasses all *Salmonella* species, including: *Salmonella typhi*, *Salmonella choleraesuis*, and *Salmonella enteritidis.* Serotypes of *Salmonella* are also encompassed herein, for example, *typhimurium*, a subgroup of *Salmonella enteritidis*, commonly referred to as *Salmonella typhimurium.*

Attenuation: Attenuation is a modification so that a bacterium or bacterial vector is less pathogenic. The end result of attenuation is that the risk of toxicity as well as other side-effects is decreased, when the bacterium or bacterial vector is administered to the patient.

Virulence: Virulence is a relative term describing the general ability to cause disease, including the ability to kill normal cells or the ability to elicit septic shock (see specific definition below).

Septic shock: Septic shock is a state of internal organ failure due to a complex cytokine cascade, initiated by TNFα. The relative ability of a bacterium or bacterial vector to elicit TNFα is used as one measure to indicate its relative ability to induce septic shock.

Gene product: Gene product refers to any molecule capable of being encoded by a nucleic acid, including but not limited to, a protein or another nucleic acid, *e.g.*, DNA, RNA dsRNAi, ribozyme, DNAzyme, *etc.* The nucleic acid which encodes for the gene product of interest is not limited to a naturally occurring full-length "gene" having non-coding regulatory elements.

Treatment: In addition to its ordinary meaning, the term treatment encompasses inhibition of progression of symptoms or amelioration of symptoms of a disease or disorder such as a solid tumor cancer.

Tumor-targeted: Tumor-targeted is defined as the ability to distinguish between a cancerous target cell or tissue and the non-cancerous counterpart cell or tissue so that tumor-targeted bacteria, such as *Salmonella* preferentially attach to, infect and/or remain viable in the cancerous target cell or the tumor environment.

Chelating agent sensitivity: Chelating agent sensitivity is defined as the effective concentration at which bacteria proliferation is affected, or the concentration at which the viability of bacteria, as determined by recoverable colony forming units (c.f.u.), is reduced.

Omp-like protein: As used herein, an Omp-like protein includes any bacterial outer membrane protein, or portion thereof (*e.g.,* signal sequence, leader sequence, periplasmic region, transmembrane domain, multiple transmembrane domains, or combinations thereof). In specific embodiments, the Omp-like protein is at least a portion of OmpA, OmpB, OmpC, OmpD, OmpE, OmpF, OmpT, a porin-like protein, PhoA, PhoE, lamB, β-lactamase, an enterotoxin, protein A, endoglucanase, peptidoglycan-associated lipoprotein (PAL), FepA, FhuA, NmpA, NmpB, NmpC, or a major outer membrane lipoprotein (such as LPP), *etc.*

Release factor: As used herein, a release factor includes any protein, or

functional portion thereof which enhances release of bacterial components. In one embodiment a release factor is a bacteriocin release protein. Release factors include, but are not limited to, the bacteriocin release protein (BRP) encoded by the cloacin D13 plasmid, the BRPs encoded by the colicin E1-E9 plasmids, or BRPs encoded by the colicin A, N or D plasmids.

Direct detection (of a marker gene product): As used herein, direct detection of a marker gene product indicates that the signal detected is a function of the gene product itself.

Indirect detection (of a marker gene product): As used herein, indirect detection of a marker gene products entails that detection of a label moiety that co-localizes with the marker gene product, for example by binding to the marker gene product or as a result of being a metabolite of a reaction catalyzed by the marker gene product.

Composition of the invention: As used herein, a composition of the invention minimally comprises a population of tumor-targeted bacteria suitable for use in the non-invasive tumor imaging or tumor imaging and treatment methods described herein. A composition of the invention optionally further comprises a population of tumor-targeted bacteria suitable for tumor therapy.

Pharmaceutical of the invention or pharmaceutical composition of the invention: As used herein, a pharmaceutical of the invention or a pharmaceutical composition of the invention refers to a composition of the invention that comprises a pharmaceutically acceptable carrier.

4. Brief Description of the Figures

The present invention may be understood more fully by reference to the following detailed description, illustrative examples of specific embodiments and the appended figures.

FIG. 1 demonstrates the ability of the imaging system to detect [^{14}C]-FIAU in tumor-bearing mice pretreated with *Salmonella* expressing HSV-TK . A tumor-implanted mouse was treated with VNP20009 expressing the plasmid p5-3 from Pawelek *et al.* (1997, Cancer Res., 57:4537-4599) four days prior to i.v. injection of labeled marker substrate ([^{14}C]-FIAU). After 23 hours, tumor tissue samples were removed from sacrificed animals and subjected to QAR. Histology of the tumor and surrounding tissue is shown on the left, the digital autoradiogram on the right and the merged image in the center. [^{14}C]-FIAU can be clearly detected within the tumor, with little or no accumulation in the surrounding tissue. See text in Section 6 for details.

FIG. 2 demonstrates the tumor-targeting selectivity of VNP20009 expressing

the plasmid p5-3. Liver tissue was collected from mice treated as described in the text in Section 6 and subjected to QAR. Histology of the tissue is shown on the left and the digital autoradiogram on the right. Little or no labeled marker substrate can be detected.

FIG. 3A shows fluorescence of an untreated tumor and figure 3B shows fluorescence observed in tumors following treatment with GFP-containing *Salmonella* strain VNP20009.

FIG. 4 shows the effect of administering non-*Salmonella* tumor-targeted bacteria on tumor growth, as a measure of tumor volume versus time following administration of the bacteria.

5. Detailed Description of the Invention

The present invention provides novel compositions and non-invasive methods of detecting tumors which exploit the tumor-specificity of tumor-targeting bacteria, as described below. Because the tumor targeting bacteria do not discriminate between different tumor types, the described compositions and methods can be used to simultaneously image multiple tumor types, in contrast to the tumor imaging reagents currently in use, which are largely specific to a single tumor type or tumor subtype.

The present invention provides a method of detecting a tumor by imaging in a subject comprising: (a) administering to the subject a tumor-targeted bacteria containing a marker gene, wherein the tumor-targeted bacteria targets the tumor cells and/or the tumor environment and the marker gene is expressed in the tumor-targeted bacteria, thereby generating a marker gene product; and (b) scanning the subject to detect the marker gene product, thereby imaging the tumor in the subject. In certain embodiments of the invention, the marker gene is detected directly. In other embodiment, the marker gene is detected indirectly. In one embodiment, indirect detection further comprises administering to the subject a labeled marker-binding moiety after step (a), wherein the marker gene product of step (a) binds to the labeled marker-binding moiety; and wherein said scanning is after clearance of residual labeled marker-binding moiety not bound to the marker gene product, thereby detecting the labeled marker-binding moiety localized to the tumor and imaging the tumor in the subject. In another embodiment, indirect detection further comprises administering to the subject a labeled marker substrate after step (a), wherein the tumor-targeted bacteria expressing the marker gene product of step (a) metabolizes the labeled marker substrate to produce a labeled marker metabolite; and wherein said scanning is after clearance of residual marker substrate not metabolized by the marker gene product, thereby detecting the labeled marker metabolite localized to the tumor and imaging the tumor in the subject.

Further, the present invention provides a method of monitoring a tumor during the course of a disease and/or treatment in a subject comprising (a) administering to the subject a tumor-targeted microorganism containing a marker gene, wherein the tumor-targeted microorganism targets the tumor cells and/or the tumor environment and the marker gene is expressed in the tumor-targeted microorganism, thereby generating a marker gene product; (b) scanning the subject to detect the marker gene product, thereby imaging the tumor in the subject; and (c) repeating steps (a) and (b) as needed during the course of the disease in the subject. The invention provides a non-invasive, clinically applicable method for imaging tumors which can be implemented using existing imaging techniques to monitor and evaluate *in vivo* cancer treatments in human subjects.

The imaging and monitoring methods of the present invention can use tumor-targeted bacterial vectors as previously described in WO 96/40238 and WO 99/13053, which are incorporated-by-reference herein in their entirety, for imaging solid tumor cancers, such as sarcomas, carcinomas, lymphomas or other solid tumor cancers, for example, renal carcinoma, mesoendothelioma, bladder cancer, germ line tumors and tumors of the central nervous system, including, but not limited to, breast cancer, prostate cancer, cervical cancer, uterine cancer, lung cancer, ovarian cancer, testicular cancer, thyroid cancer, astrocytoma, glioma, pancreatic cancer, stomach cancer, liver cancer, colon cancer, melanoma. The novel imaging, monitoring, detecting and treatment methods of the present invention can be used with existing nuclear medicine instrumentation as previously described in United States Patent No. 5,703,056 entitled "Non-Invasive Imaging of Gene Transfer" issued December 30, 1997, which is incorporated-by-reference herein in its entirety.

In various embodiments, the present invention provides a means of imaging or monitoring tumors by delivering high levels of the products of marker gene(s), either alone or together with therapeutic gene(s), using various tumor-targeted strains of bacteria (preferably modified and/or attenuated) which selectively accumulate at or within tumors while expressing the marker gene(s) and/or therapeutic genes.

When administered to a subject, *e.g.*, an animal for veterinary use or to a human for clinical use, the vectors can be used alone or may be combined with any physiological carrier such as water, an aqueous solution, normal saline, or other physiologically acceptable excipient. In general, the dosage would range from about 1 to $1x10^9$ c.f.u./kg, preferably about 1 to $1x10^2$ c.f.u/kg.

The vectors of the present invention can be administered by a number of routes, including but not limited to: orally, topically, injection including, but limited to intravenously, intraperitoneally, subcutaneously, intramuscularly, intrathecally,

intratumorally, *i.e.*, direct injection into the tumor, *etc.*

In certain embodiments, the imaging methods of the invention are used to monitor the efficiency of vector targeting prior to administration of a suicide substrate. In these embodiments, visualization of a bacterial vector at a tumor site serves the purpose of identifying the tumor site and provides an indication of whether sufficient vector targeting to a tumor has indeed taken place. In a preferred embodiment, a single tumor-targeted bacterial vector contains both a marker gene and a suicide gene. In another preferred embodiment where imaging is achieved by administering a labeled compound that is incorporated into the bacteria, the bacterial vector harbors a mutation that increases incorporation of the labeled compound into the bacteria and contains a suicide gene. In another preferred embodiment, two tumor-targeted bacterial vectors are administered, each vector containing either a marker gene or a suicide gene. After administration of one or more vectors, administration of a labeled marker substrate, compound or binding moiety for imaging is performed. This permits assessment of the specificity of the vector for the tumor by the practitioner. Further, the amount of vector targeted to the tumor can be determined by correlating the intensity of the image with the amount administered. If it is determined that sufficient amount of vector has been delivered with sufficient specificity, suicide substrate can then be administered to treat the tumor. For further details relating to this embodiment, see Section 5.4., *infra*.

In other embodiments, the invention provides methods for imaging or monitoring a tumor while simultaneously treating the tumor using one or more of the tumor-targeting bacterial vectors described and referenced herein. For example, a subject may be diagnosed with a solid tumor cancer by any method known in the art. The vector, *i.e.*, the tumor-targeted bacteria, used in simultaneous imaging or monitoring and treatment may be isolated using the methods of the present invention with target cell lines or using model tumors in mice. In another embodiment, a biopsy of tumor cells is used in a selection assay for isolating a vector which is super-infective and tumor-specific for the tumor of the subject. In a preferred embodiment, the vector used is genetically modified to express, for example, a suicide gene or to delete virulence factors, or both, as described herein. In addition, the isolated vector may be analyzed for sensitivity to antibiotics to insure the eradication of the vector from the patient's body after successful treatment or if the patient experiences complications due to the administration of the vector.

In a highly preferred embodiment, the methods of *in vivo* imaging and treatment are combined, wherein the imaging and treatment vectors are separate tumor-targeted bacterial vectors. In one embodiment, each vector is capable of expressing a mutant gene selected for substrate specificity, wherein the imaging mutant gene is retained

in the cytosol of a first tumor-targeted bacterial vector and the treatment mutant gene is in the periplasm of a second tumor-targeted bacterial vector. In another embodiment, a single vector contains both a suicide gene and an imaging gene, where the suicide gene and imaging gene are carried on different plasmids or are incorporated into bacterial DNA. In another embodiment, different mutants of HSV1-TK or VZV-TK serve as both the suicide gene and the imaging gene in the same tumor-targeted bacterial vector.

In an embodiment where the suicide gene and the imaging gene are carried in two different vectors administered simultaneously to a subject, preferably the two different vectors are produced from the same bacterial strain and exhibit the same tumor-specificity. The two different vectors may also be administered sequentially within a short time of one another, and in any order.

It will be understood by one of ordinary skill in the art that the imaging instrumentation, such as γ camera or single-photon emission computed tomography (SPECT), PET, MRI, *etc.*, and the corresponding relevant labeled marker substrates as described in United States Patent No. 5,703,056 entitled "Non-Invasive Imaging of Gene Transfer" issued December 30, 1997 to Blasberg and Tjuvajev, which patent is incorporated-by-reference herein in its entirety, can be used in the novel imaging, monitoring, detecting and treatment methods of the present invention.

Subjects are scanned using such technologies by methods well known to those skilled in the art. Standard nuclear medicine imaging equipment is employed.

5.1. Vectors for Imaging or Monitoring a Tumor

In a preferred embodiment of the invention, tumor-targeted bacteria (see Section 5.5 *infra*) are engineered to become bacterial vectors of the invention to express one or more marker genes (optionally, including one or more therapeutic genes) suitable for imaging or monitoring (or optionally, including treating) a tumor.

In another preferred embodiment of the invention, tumor-targeted bacteria are administered in conjunction with a labeled compound that is incorporated into the bacteria for tumor imaging, monitoring, and, optionally, treatment.

It will be understood by one skilled in the art that imaging a tumor using the methods of the invention is equivalent to tumor detection.

In an exemplary embodiment, a marker gene is a gene coding for an enzyme that may be expressed in the tumor-targeted bacterial vector and that catalyzes a reaction with a labeled marker substrate, resulting in the accumulation of a detectable marker within the vector. The herpes simplex virus thymidine kinase gene (HSV1-tk) and the varicella zoster virus thymidine kinase gene (VZV-tk) are preferred "marker genes" and a labeled 2-

'fluoro-nucleoside analogue is their preferred marker substrate. More specifically, 2'-fluoro-5-iodo-1-β-D-arabinofuranosyl- uracil (FIAU) is a preferred "marker substrate" for the enzyme HSV1 thymidine kinase (HSV1-TK), the gene product of HSV1-tk and for the enzyme the varicella zoster virus thymidine kinase (VZV-TK), the gene product of VSV-tk. In another embodiment, a preferred marker substrate is 2'-fluoro-5-iodo-1-β-D-ribofuranosyl- uracil. For additional labeled marker substrates useful when the marker gene is tk, see *infra* at Section 5.2. Other self-complementary pairs of marker genes and labeled marker substrates are also discussed *infra* at Section 5.2. In a preferred mode of the exemplary embodiment, the HSV1-TK- or VZV-TK-expressing vector is administered to the subject concurrently with a pro-drug (*e.g.* ganciclovir or acyclovir). The pro-drug is phosphorylated in the periplasm of the microorganism which is freely permeable to nucleotide triphosphates. The phosphorylated ganciclovir or acycolvir, toxic false DNA precursors, readily pass out of the periplasm of the microorganism and into the cytoplasm and nucleus of the host cell where they incorporate into host cell DNA, thereby causing the death of the host cell.

In a highly preferred embodiment, the marker gene product of a tumor-targeted bacterial vector is retained in the cytosol of the vector, where such vector is used for imaging or monitoring a tumor. Marker gene product retention in the cytosol of a tumor-targeted bacterial vector aids in the imaging or monitoring of particularly small, difficult-to-detect tumors (*i.e.* less than one gram) by maximizing marker gene product accumulation at the tumor site. Accordingly, tumor-targeted bacterial vector expression constructs are preferably designed such that a marker gene product (*e.g.*, a thymidine kinase enzyme, a glucokinase enzyme, or a cytochrome P-450 enzyme) is not secreted from the bacterial vector. In another embodiment, the marker gene product of a tumor-targeted bacterial vector is retained in the cytosol and periplasm of the vector, where such vector is used for imaging or monitoring a tumor. In another embodiment, the marker gene product of a tumor-targeted bacterial vector is retained in the periplasm of the vector, where such vector is used for imaging or monitoring a tumor.

In another embodiment of the invention, a vector containing a marker gene for imaging a tumor is under the specific regulatory control of certain types of promoters. These promoters may be either constitutive, in which the genes are continually expressed, inducible, in which the genes are expressed only upon the presence of an inducer molecule(s) or cell-type or tumor-environment specific control, in which genes are expressed only or preferentially in certain cell types or only in the tumor-environment, respectively. In one embodiment, for example, the expression of the gene is controlled by a bacterial promoter which may be activated by secretions or other molecules specific to a

given tumor. In a preferred embodiment, the bacterial promoter is activated only by secretions or other molecules specific to a given tumor.

5.2. Methods for Detecting or Monitoring by Imaging

The detecting and monitoring compositions and/or methods of the present invention allow for the localization and identification of tumors for which whole body detection methods are unavailable or impractical. The detecting and monitoring compositions and/or methods of the present invention further allow for the localization and identification of tumors smaller than those diagnosable by methods available prior to the invention. Generally, prior methods are limited to detection of a tumor having at least one gram in mass. Accordingly, in one embodiment, a tumor having less than one gram mass is detected or monitored. In another embodiment, a tumor from 0.99 grams to 0.01 grams mass is detected or monitored. In yet another embodiment, a tumor from 0.9 grams to 0.1 grams mass is detected or monitored. In yet still another embodiment, a tumor from 0.8 grams to 0.2 grams mass is detected or monitored. In a preferred embodiment, a tumor of from about 0.05 to about 0.1 grams mass is detected or monitored (plus or minus twenty percent).

In another embodiment, the imaging methods of the present invention are used to monitor the progression of a tumor over time. In this embodiment, one or more tumors are visualized according to the methods of the invention over time, thereby allowing detection of any changes in size, shape and/or location of tumors being monitored. In another embodiment, the efficacy of an anti-cancer treatment, such as chemotherapy, is monitored by visualizing the tumor before, during and/or after treatment. Suppression of tumor growth or shrinkage or disappearance of tumors during monitoring is indicative of a successful course of treatment.

In yet another embodiment, the imaging methods of the invention are used to monitor the efficiency of vector targeting. In this embodiment, visualization serves the purpose of identifying the location of the vector to determine whether sufficient vector targeting to a tumor has taken place. In a particular embodiment, a single tumor-targeted bacterial vector contains both a marker gene and a therapeutic gene. In another particular embodiment, separate vectors each containing either a marker gene or a therapeutic gene are used. In one embodiment, after administration of the vector(s), administration of marker substrate and imaging is performed to assess the specificity of the vector for the tumor and the amount of vector targeted to the tumor. If it is determined that sufficient amount of vector has been delivered with sufficient specificity, suicide substrate can be administered to treat the tumor. In a preferred embodiment, the marker gene and the suicide gene are the

same gene, *e.g.*, HSV1-TK or VZV-TK. In another preferred embodiment, the suicide gene and the marker gene are mutants of the same gene selected for marker substrate specificity and suicide substrate specificity, respectively. In a preferred embodiment, when two vectors are used, the different vectors, each containing either a suicide gene or a marker gene, are produced from the same strain of bacteria so that they have similar or identical targeting characteristics.

In another embodiment, a tumor-targeted bacterial vector used for *in vivo* imaging according to the methods of the invention may be attenuated such that, when administered to a subject, the vector is less toxic to the subject and easier to eradicate from the subject's system. In a specific embodiment, such a vector is super-infective and specific for a target tumor. In a more preferred embodiment, such a vector is also sensitive to a broad range of antibiotics.

In another embodiment, a tumor-targeted bacterial vector carrying a gene for imaging also carries a gene, such as a gene encoding a pro-drug converting enzyme, which is expressed and secreted by the vector in or near the target tumor. Such a gene can be under the control of either constitutive, inducible or tumor cell-type or tumor-environment specific promoters. In a preferred embodiment, such a gene is expressed and secreted only when a vector has invaded the cytoplasm of a target tumor cell, thereby limiting the effects due to expression of the gene to the target site of the tumor.

5.2.1 Direct Detection of Marker Gene Products

In certain embodiments of the present invention, the marker gene expressed by the bacterial vector encodes a directly detectable marker gene product. Such a marker gene can be a gene encoding a fluorescent protein, a bioluminescent protein, or a chemiluminescent protein.

In a preferred embodiment, the marker gene encodes a fluorescent molecule. In a preferred mode of the embodiment, the fluorescent molecule is firefly luciferase. In another preferred mode of the embodiment, the protein encoded by the marker gene is GFP from *Aequorea victoria* or a mutant thereof.

The GFP expressed in a tumor-targeted bacteria for tumor imaging according to the methods of the invention can be encoded by its naturally occurring coding sequence or by a coding sequence that has been modified for optimal bacterial codon usage. Mutations can be introduced into the coding sequence to produce GFP mutants with altered fluorescence wavelength or intensity or both. Such mutations are largely in the vicinity of residues 65-67, which form the chromophore of the protein. Examples of useful GFP mutations for use as marker genes according to the methods of the present invention can be

found in U.S. Patent Nos. 5,777,079 and 5,804,387 and International Publication WO97/11094. In another preferred mode of the embodiment, the GFP mutant is a blue GFP. Examples of blue GFPs are described by Heim and Tsien (1996, Curr. Biol. 6:178-82). In yet another preferred mode of the embodiment, the fluorescent protein is a yellow or red-orange emitter recently discovered in reef corals (Matz *et al.*, 1999, Nature Biotechnol. 17:969-973). Whole-body imaging of GFP-expressing tumors and metastases has been reported by Yang *et al.* (2000, Proc. Nat'l Acad. Sci. U S A 97:1206-11).

5.2.2 Detection of Marker Gene Products via a Labeled Marker-binding Moiety

In certain embodiments of the present invention, a marker gene product can be detected indirectly. For example, a marker gene product can be detected by administration of a labeled moiety that binds to the marker gene product. As used herein, binding of a labeled moiety to a marker gene product encompasses protein-protein interactions, protein-compound interactions, protein-peptide interactions, enzyme-substrate interactions, and chelating activity. The labeled moiety can be a proteinaceous or non-proteinaceous molecule, *e.g.*, a carbohydrate. A proteinaceous marker-binding moiety can be a ligand, an antibody, or any other peptide or polypeptide that interacts specifically with, and has a high affinity to, the marker gene product.

Accordingly, as described above, the invention provides indirect methods of imaging a tumor in a subject comprising administering to the subject a tumor-targeted bacteria containing a marker gene, wherein the tumor-targeted bacteria targets the tumor cells and/or the tumor environment and the marker gene is expressed in the tumor-targeted bacteria, thereby generating a marker gene product; administering to the subject a labeled marker-binding moiety, wherein the marker gene product binds to the labeled marker-binding moiety; and scanning the subject to detect the marker gene product, wherein said scanning is after clearance of residual labeled marker-binding moiety not bound to the marker gene product, thereby detecting the labeled marker-binding moiety localized to the tumor and imaging the tumor in the subject.

In certain embodiments of the present invention, the marker gene expressed by the bacterial vector is avidin or streptavidin. These proteins bind biotin with high affinity (Kendrew , ed, The Encyclopedia of Molecular Biology, 1994, Blackwell Science Ltd., pps. 80 and 1037). Streptavidin, a protein from *Streptomyces avidinii*, can chelate biotin as well as other compounds (*e.g.*, boron, see Sano, 1999, Bioconjug Chem. 10:905-911). The accumulation of chelated detectable agents, such as labeled biotin, can be detected by various methods and thus be used to facilitate the localization and monitoring of a tumor.

In other embodiments of the present invention, the marker gene expressed by the bacterial vector encodes an enzyme (*e.g.*, bacterial lacZ protein or chloramphenicol acetyl transferase (CAT)). An enzyme encoded by a marker can be detected by the administration of a labeled substrate analog.

In yet other embodiments of the present invention, the marker gene expressed by the bacterial vector encodes a receptor, or a protein comprising the ligand binding domain of a receptor, for example a fusion protein comprising the ligand-binding domain of the receptor and a protein which targets the ligand-binding domain to the outer bacterial membrane. In one embodiment, the receptor is a nicotinic acetylcholine receptor, which can be imaged by PET and SPECT using radiolabeled nicotine analogs. For example, halogenated 3-pyridyl ether compounds can be used for imaging the alpha 4 beta 2 nicotinic acetylcholine receptor (Gundisch, 2000, Curr Pharm Des 6(11):1143-57). In other embodiments, the receptor is a dopamine or serotonin receptor.

In yet other embodiments of the present invention, the marker gene expressed by the bacterial vector is, or a protein comprising an immunologically detectable epitope or other binding moiety (*e.g.*, myc, glutathione-S-transferase (GST), or hexahistidine). To facilitate easier detection of the marker gene product with reduced background signal levels, the marker gene encodes a protein that is either expressed at very low levels in the subject or, most preferably, an exogenous protein.

5.2.3 Detection of Marker Gene Products via Production of a Labeled Marker Metabolite

When the marker gene product possesses catalytic activity, the gene product can be detected indirectly by virtue of its activity, for example by production of a detectable metabolite following a chemical modification of a substrate molecule. Accordingly, preferred marker genes to practice the present embodiments are enzymes.

Accordingly, as described above, the invention provides an indirect method of tumor imaging comprising administering to the subject a tumor-targeted bacterial vector containing a marker gene, wherein the tumor-targeted bacterial vector targets to the tumor and/or infects cells of the tumor, under conditions in which the marker gene is expressed in the tumor-targeted bacterial vector, thereby generating a marker gene product; administering to the subject a labeled marker substrate under conditions in which the labeled marker substrate is metabolized by the marker gene product to produce a labeled marker metabolite which is substantially retained in the tumor-targeted bacterial vector throughout a time-period sufficient for imaging the labeled marker metabolite; and scanning the subject to detect the labeled marker metabolite, thereby imaging and detecting a tumor in the subject.

In certain specific embodiments, the generation of the marker metabolite from the marker substrate entails the release of a quenched label from the substrate (*see, e.g.*, Bell and Taylor-Robinson, 2000, Gene Therapy 7:1259-1264).

Suitable enzymes that can produce detectably labeled metabolites include but are not limited to malate dehydrogenase, staphylococcal nuclease, δ-5-steroid isomerase, yeast alcohol dehydrogenase, α-glycerophosphate, dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, β-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholinesterase. In one embodiment, the tumor-targeted bacteria also express a permease or transporter which allows for enhanced uptake of the substrate of the marker gene product enzyme. In an exemplary mode of the embodiment, the tumor-targeted bacteria that express lacZ would also express lacY to increase lactose or galactose uptake.

In other embodiments, suitable enzymes that can produce detectably labeled metabolites are proteases. Suitable protease substrates for imaging are polymerized protease recognition sites bearing a quenched label, which have been called "repeating graft co-polymers" (Weissleder *et al.*, 1999, Nature Biotechnology 17:375-378). Cleavage of the substrate by the recombinant protease expressed by the tumor-targeted vector at the tumor site releases the quenched label, *i.e.*, generates a labeled metabolite, allowing imaging of the tumor. Synthesis of quenched substrates can be done according to the principles described in Weissleder *et al.* (1999, Nature Biotechnology 17:375-378).

In a preferred embodiment, the marker gene expressed by the vector is a wild type, natural mutant or genetically-engineered HSV1-TK or VZV-TK gene, and its substrate is a labeled nucleoside analog such as FIAU, ACV or GCV. In a particular mode of the embodiment, the nucleoside analog is a 2'-fluoro-nucleoside analogue such as FIAU, . Upon concurrent expression of the tk gene and administration of radioactively labeled FIAU to the subject, the FIAU is phosphorylated in the microorganism. The phosphorylated FIAU accumulates in the microorganism. This accumulated, labeled FIAU is then imaged by scanning the subject using, for example, positron emission tomography, γ camera or single-photon emission computed tomography. In a highly-preferred embodiment, the FIAU is phosphorylated in the cytosol of the microorganism instead of the periplasm to enhance accumulation and subsequent imaging. This may be accomplished using a vector genetically engineered to express TK in the cytosol of the microorganism.

In certain modes of the embodiment in which the marker gene is TK, the labeled 2'-fluoro-nucleoside analogue is 5-[^{123}I]-, 5-[^{124}I]- or 5-[^{131}I]-2'-fluoro-5-iodo-1-ß-D-arabinofuranosyl- uracil; 5-[^{18}F]-2'-fluoro-5-fluoro-1-ß-D- arabinofuranosyl- uracil; 2-[^{11}C]-

or 5-([^{11}C]-methy)-2'-fluoro-5-methyl-1-ß-D-arabinofuranosyl- uracil; 2-[^{11}C]- or 5-([^{11}C]-ethyl)-2'-fluoro-5-ethyl-1-ß-D-arabinofuranosyl- uracil; 5-(2-[^{18}F]-ethyl)-2'-fluoro-5-(2-fluoro-ethyl)-1-ß-D-arabinofuranosyl- uracil; 5-[^{123}I]-, 5-[^{124}I]- or 5-[^{131}I]-2'-fluoro-5-iodovinyl-1ß-D-arabinofuranosyl- uracil; 5-[^{123}I]-, 5-[^{124}I]- or 5-[^{131}I]-2'- fluoro-5-iodo-1-ß-D-arabinofuranosyl- uracil; or 5-[^{123}I]-, 5-[^{124} I]- or 5-[^{131}I]-2'-fluoro-5-iodovinyl-1-ß-D-aribofuranosyl- uracil.

In another preferred embodiment, the marker gene expressed by the vector is wild type, natural mutant or genetically-engineered yeast glucokinase gene, and its substrate is a labeled 3-O-methyl glucose. In certain modes of the embodiment, the labeled 3-O-methyl glucose is [^{11}C]- or [^{18}F]-3-O-methyl glucose.

In yet another preferred embodiment, the marker gene expressed by the vector is wild type, natural mutant or genetically-engineered cytochrome P-450 B1 gene, and its substrate is a labeled imidazole substrate. In certain modes of the embodiment, the labeled imidazole substrate is 2-[^{11}C]-misonidazole, 2-[^{11}C]-metronidazole or 3-[^{18}F]-fluoromisonidazole.

5.2.4 Detection of Labeled Compounds Incorporated Into Bacteria

The invention also encompasses the detection of a tumor by administering to the subject in which tumor detection is desired tumor-targeted bacteria. The tumor targeted bacteria is then detected by administering to the subject a labeled compound which is incorporated or localized to the bacteria itself. For example, the labeled compound can be chosen to be incorporated into the bacterial cell wall. The labeled compound is labeled with one of the labels described in Section 5.3, *infra* and detected as described.

In one embodiment, the labeled compound is labeled diaminopimelic acid (DAP), as described below. In another embodiment, the labeled compound is a labeled glucose or glucose analog which, because of the higher proliferation rate of the bacteria (as well as the tumor cells) relative to normal mammalian cells, will be preferentially incorporated into the dividing bacteria and tumor cells. This improves on the current imaging systems in which labeled glucose analogs are used to image tumors by enhancing the specific signal and reducing the non-specific signal, allowing the detection of smaller tumors, including metastases. In a preferred embodiment, the labeled glucose analog is ^{18}F-fluorodeoxyglucose (^{18}F-FDG), which can be detected by PET scanning.

In certain embodiments, the bacteria's metabolism is manipulated to increase its incorporation of the labeled compound. In one embodiment, the bacteria has an auxotrophic mutation which enhances its uptake of the labeled compound. Preferably, such a mutation further compromises its ability to live independently of the compound, such that

its viability is largely dependent on the administration of the compound. Following administration of the compound and imaging the bacteria (and, indirectly, the tumor(s)), such bacteria are gradually cleared from the system.

In one mode of the embodiment, the tumor-targeted bacteria is mutant for the *asd* locus, which makes the bacteria more susceptible to lysis due to compromised cell wall integrity. Rescue of this mutant can be achieved by exogenously providing DAP(Galan *et al.*, 1990, Gene 94:24-35). When labeled DAP is provided to bacteria with an *asd* mutation, the DAP becomes incorporated into the cell wall. Therefore, administering labeled DAP to a subject to whom *asd*$^+$ tumor-targeting bacteria have been administered enhances the incorporation of DAP into the cells walls of the bacteria, resulting in an enhanced signal for imaging relative to the use of non-*asd*$^+$ tumor-targeting bacteria. Additionally, depletion of DAP from the subject eventually results in bacterial death, thereby clearing the bacteria from the subject after imaging. In a preferred mode of the embodiment, the *asd*$^+$ mutation is a hypomorphic but not a null mutation, so that the bacteria can survive administration and targeting to the tumor prior to administration of the labeled DAP.

5.2.5 Detection of Bacterial Infections

In certain embodiments of the invention, a tumor is detected by detecting an infection (*e.g.*, the immune response) caused by administering a composition of the invention to a subject.

In one embodiment, tumor-targeted bacteria are administered to a subject and the subject scanned to detect an infection caused by the bacteria at the tumor site. The infection, and therefore the tumor, is detected indirectly by detecting the presence of an antigen that is highly or specifically expressed at the infection site. In a preferred mode of the embodiment, the antigen detected is a neutrophil-specific antigen. Polymorphonuclear neutrophils are sequestered at infection sites and labeled monoclonal neutrophil-specific antigens have been successful to diagnose infections. In a preferred mode of the embodiment, the neutrophil-specific antigen is CD15. A ^{99}Tc-labeled anti-CD15 monoclonal antibody is commercially available under the trade name LeuTech (Palatin Technologies, Inc.), which has been used to detect appendicitis (Kipper *et al.*, 2000, J Nucl Med 41(3):449-55).

In another embodiment, the infection, and therefore the tumor, is detected indirectly by using a labeled chemotactic peptide analogs which bind to leukocyte receptors (*see, e.g.*, Fischman *et al.*, 1994, Semin Nucl Med). In one mode of the embodiment, the labeled chemotactic peptide analog is labeled N-formyl-methionyl-leucyl-phenylalanine (ForMLF). In another mode of the embodiment, the labeled chemotactic peptide analog is

labeled neutrophil peptide-1. ^{99}Tc-labeled neutrophil peptide-1 has been used to detect bacterial infections (Welling *et al.*, 1999, J Nucl Med 40(12):2073-80).

5.2.6 Detection of Bacterial Surface Antigens

In certain embodiments of the invention, a tumor is detected by detecting a surface antigen expressed by the tumor-targeted bacteria.

According to the present embodiment, tumor-targeted bacteria are administered to a subject. A labeled antibody which binds to an antigen present on the surface of the tumor-targeted bacteria is then administered, and the subject scanned to detect the label. Therefore the tumor is detected indirectly by detecting the presence of an antigen that is expressed on the bacterial surface.

In one embodiment, the surface antigen detected by the antibody is an O-antigen (an abbreviation for O-polysaccharide antigen). In another embodiment, the surface antigen detected by the antibody is an H-antigen (H-antigens are flagellar antigens). In yet another embodiment, the surface antigen detected by the antibody is an outer membrane protein.

5.3. Labels and Methods of Detection Thereof

The present invention provides non-invasive methods of imaging tumors *in vivo* by detecting tumor-targeted bacteria or a marker gene product expressed by a tumor-targeted bacteria, said detection being by way of a labeled moiety, metabolite, or compound. Some examples of labels that can be used are radioisotopes (such as ^{3}Hydrogen, ^{11}Carbon, ^{14}Carbon, ^{13}Nitrogen, ^{18}Flourine, ^{123}Iodine, ^{124}Iodine, ^{125}Iodine, ^{131}Iodine, ^{111}Indium, ^{64}Copper, ^{67}Copper, ^{43}Scandium, ^{44}Scandium, ^{46}Scandium, ^{47}Scandium, ^{48}Scandium, ^{72}Gallium, ^{73}Gallium, ^{203}Bismuth, ^{205}Bismuth, ^{212}Bismuth, ^{213}Bismuth, ^{214}Bismuth, ^{52}Iron, ^{55}Cobalt, ^{68}Gallium.); paramagnetic metal ions (such as ^{54}Iron, ^{56}Iron, ^{57}Iron, ^{58}Iron, ^{157}Gadolinium, ^{55}Manganese); and ferromagnetic metals (such as magnetite (Fe_3O_4), γ-ferric oxide (γ-Fe_2O_3), cobalt ferrite, nickel ferrite, manganese ferrite). Label detection can be performed with γ camera or single-photon emission computed tomography (SPECT), PET, and MRI.

Fluorophores can also be used as labels for marker detection. Examples of such compounds are rhodamine, fluorescein, Cy5, Cy3, succinimidyl 6-(N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl)amino)hexanoate (also known as NBT), R-phycoerythrin, allophycocyanin, 6-((7-amino-4-methylcoumarin- 3-acetyl)amino)hexanoic acid (also known as AMCA), 7-dimethylaminocoumarin-4- acetic acid (also known as DMACA), and 5-carboxytetramethylrhodamine. Label detection can be performed with a

whole-body optical fluorescence imaging system (Yang *et al.*, 2000, Proc. Nat'l Acad. Sci. U S A 97:1206-11).

The particular label, optimal label position and method of labeling for each labeled moiety, substrate or compound that is administered to a subject for imaging or a combination of imaging and treatment will depend on the chemical nature of the moiety, substrate or compound and its intended use (imaging only or imaging and therapeutic use) and can be determined by one of skill in the art.

In certain specific embodiments, the label is chelated or quenched until it reaches the tumor site, where the label is released. Both fluorophores (Weissleder *et al.*, 1999, Nature Biotechnology 17:375-378) and paramagnetic ions (Bell and Taylor-Robinson, 2000, Gene Therapy 7:1259-1264), which have been successfully used as quenched labels that are eventually released to produce a signal, can be used according to the present invention.

5.4. Improving the Signal to Noise Ratio

The present invention further provides methods by which the signal of the label or the marker gene product to be imaged can be enhanced. Such methods encompass enhancing the specific signal, reducing the background or non-specific signal, or both.

In one embodiment, the invention provides a method further comprising waiting a time-period after administration of the labeled compound, substrate or binding moiety sufficient to allow at least 67% of non-specific label to clear from the subject. In another embodiment, the invention provides a method further comprising waiting a time-period after administration of the labeled compound, substrate or binding moiety sufficient to allow at least 80% of non-specific label to clear from the subject. In yet another embodiment, the invention provides a method further comprising waiting a time-period after administration of the labeled compound, substrate or binding moiety sufficient to allow at least 90% of non-specific label to clear from the subject. As used herein, the term non-specific label refers to labeled (i) compound, (ii) substrate or (iii) binding moiety that (i) has not been incorporated into the bacteria, (ii) has not been metabolized by the marker gene product, or (iii) is not bound to the marker gene product, respectively.

In certain other embodiments of the invention, the label in a labeled substrate is quenched or chelated for release at a tumor site upon production of a labeled metabolite. The labeled substrate is undetectable or generates a reduced signal until the label is activated, thereby reducing the non-specific signal that could be generated from non-specific binding or poor clearance of the substrate.

In yet other embodiments of the invention, the specific signal is enhanced by

maximizing the interaction of the marker gene product with the labeled compound, substrate or binding moiety. In certain specific embodiments, as described below, the marker gene product is brought into the vicinity of the labeled compound, substrate or binding moiety, for example by promoting its release into the extracellular environment. In other specific embodiments, as described below, the labeled compound, substrate or binding moiety is brought into the vicinity of the marker gene product, for example by promoting its uptake into or retention in the bacterial vector.

5.4.1 Non-Selective Increase in Bacterial Release of Intracellular Compounds

In certain embodiments of the invention, the attenuated tumor-targeted bacterial vectors of the invention, which express at least one marker gene product, also express at least molecule which functions to permeabilize the bacteria cell membrane(s) or enhance the release of intracellular components into the extracellular environment, *e.g.* at the tumor site, thereby enhancing the delivery of the marker gene product(s). Such molecules which permeabilize the bacterial cell or enhance release are designated as "release factors".

The release factor expressed by the bacterial vector of the invention may be endogenous to the modified attenuated tumor-targeted bacteria or it may exogenous (*e.g.*, encoded by a nucleic acid that is not native to the attenuated tumor-targeted bacteria). A release factor may be encoded by a nucleic acid comprising a plasmid, or by a nucleic acid which is integrated into the genome of the attenuated tumor-targeted bacteria. A release factor may be encoded by the same nucleic acid or plasmid that encodes the marker gene of interest, or by a separate nucleic acid or plasmid..

In a preferred embodiment, such a factor is one of the Bacteriocin Release Proteins, or BRPs (herein referred to in the generic as BRP). The BRP employed in the invention can originate from any source known in the art. Examples of BRP proteins include, but are not limited to, the BRP from the cloacin D13 plasmid, the BRP from one of the colicin E1-E9 plasmids, and BRP from colicin A, N or D plasmids. In a preferred embodiment, the BRP is of cloacin D13 (pCloDF13 BRP).

In another embodiment of the invention, the enhanced release system comprises overexpression of a porin protein; *see e.g.*, Sugawara and Nikaido, 1992, J. Biol. Chem.267:2507-11).

Expression levels of release factors and porins should be optimized to enhance release of cellular contents but not result in bacterial lysis. Preferably, such expression is under the control of a promoter that is preferentially active in the tumor environment. For example, the release factor or porin coding sequence can be expressed

under the control the pepT promoter, which is activated in response to the anaerobic nature of the tumor environment (see, *e.g.*, Lang *et al.*, 1996, Gene, 168:169-171). In another specific embodiment of the invention, the release factor or porin coding sequence is expressed under the control of a *tet* promoter, and expression of the release factor or porin is induced upon administration of tetracycline to the subject.

5.4.2 Targeted Marker Gene Release

The present invention also provides methods for local delivery of one or more marker gene products to the tumor environment through secretion machinery. In a preferred embodiment, tumor-targeted bacteria are engineered to express one or more nucleic acid molecules encoding one or more fusion proteins comprising an Omp-like protein, or portion thereof (*e.g.*, signal sequence, leader sequence, periplasmic region, transmembrane domain, multiple transmembrane domains, or combinations thereof; see Section 3.1 for definition of "Omp-like protein") and marker gene product.

In specific embodiments, the Omp-like protein is at least a portion of OmpA, OmpB, OmpC, OmpD, OmpE, OmpF, OmpT, a porin-like protein, PhoA, PhoE, lamB, β-lactamase, an enterotoxin, protein A, endoglucanase, peptidoglycan-associated lipoprotein (PAL), FepA, FhuA, NmpA, NmpB, NmpC, or a major outer membrane lipoprotein (such as LPP), *etc.* In certain embodiments of the invention, the signal sequence is constructed to be more hydrophobic (*e.g.*, by the insertion or replacement of amino acids within the signal sequence to hydrophobic amino acids, *e.g.*, Leucine).

In several embodiments, the marker gene product is expressed as a fusion protein to an Omp-like protein, or portion thereof. Bacterial outer membrane proteins are integral membrane proteins of the bacterial outer membrane, possess multiple membrane-spanning domains and are often attached to one or more lipid moieties. Outer membrane proteins are initially expressed in precursor form (the pro-Omp) with an amino terminal signal peptide that directs the protein to the membrane, upon which the signal peptide is cleaved by a signal peptidase to produce the mature protein. In one embodiment, a marker gene product is constructed as a fusion protein with an Omp-like protein. In this embodiment, the gene product has enhanced delivery to the outer membrane of the bacteria.

In one embodiment, the fusion of a marker gene product to an Omp-like protein is used to enhance localization of marker gene product to the periplasm. In another embodiment, the fusion of a marker gene product to an Omp-like protein is used to enhance release of the gene product. In another embodiment, a release factor (*e.g.*, BRP, *supra*) is expressed in a cell which also expresses a fusion protein comprising a marker gene product fused to an Omp-like protein. In this embodiment, the co-expression of the release factor

allows for enhanced release of the fusion protein from the periplasmic space.

In other embodiments of the invention, a fusion protein of the invention comprises a proteolytic cleavage site. The proteolytic cleavage site may be endogenous to the gene product or endogenous to the Omp-like protein, or the proteolytic cleavage site may be constructed into the fusion protein. In certain specific embodiments, the Omp-like protein of the invention is a hybrid Omp comprising structural elements that originate from separate proteins.

In an exemplary mode of the embodiment, the Omp-like protein is OmpA; the same principles used in the construction of OmpA-like fusion proteins are applied to other Omp fusion proteins, keeping in mind the structural configuration of the specific Omp-like protein.

For example, the native OmpA protein contains eight anti-parallel transmembrane β-strands within the 170 amino acid N-terminal domain of the protein. Between each pair of transmembrane domains is an extracellular or intracellular loop, depending on the direction of insertion of the transmembrane domain. The C-terminal domain consists of 155 amino acids which are located intracellularly and presumably contact the peptidoglycan occupying the periplasmic space. Expression vectors have been generated that facilitate the generation of OmpA fusion proteins. For example, Hobol *et al.* (1995, Dev. Biol. Strand. 84:255-262) have developed vectors containing the OmpA open reading frame with linkers inserted within the sequences encoding the third or fourth extracellular loops that allow the in-frame insertion of the heterologous protein of choice.

In another embodiment of the invention, the portion of the OmpA fusion protein containing the marker gene product is at the extracellular bacterial surface. In one aspect of the embodiment, the fusion protein comprises an even number or odd number of membrane-spanning domains of OmpA located N-terminal to the gene product. In another aspect of the embodiment, the gene product is situated between two extracellular loops of OmpA for presentation to the tumor cell by the bacterial cell. In specific embodiments, the invention provides expression plasmids of gene product fusion proteins at the bacterial extracellular surface. For example, the plasmid denoted Trc(lpp)*ompA*, comprises a *trc* promoter-driven lipopolyprotein (lpp) anchor sequence fused to a truncated *ompA* transmembrane sequence. As another example, the plasmid is denoted Trc*ompA* comprises a *trc* promoter-driven *ompA* gene signal sequence. Such plasmids may be constructed to comprise a nucleic acid comprising or encoding one or more gene product(s) of the invention.

Optionally, a marker gene product is preceded or flanked by consensus cleavage sites for a metalloprotease or serine protease that is abundant in tumors, for release

of the gene product into the tumor environment. Whether the marker gene product is preceded or flanked by protease cleavage sites depends on whether it is located terminally or internally in the fusion protein, respectively.

Similar fusion proteins may be constructed with any of the Omp-like proteins using the strategies described above in terms of OmpA. In the construction of such fusion proteins, as will be apparent to one of ordinary skill in the art, the selection of the portion of the Omp-like protein to be fused to a marker gene product will depend upon the location that is desired for the expression of the gene product (*e.g.* periplasmic, extracellular, membrane bound, *etc.*).

Construction of fusion proteins for expression in bacteria are well known in the art and such methods are within the scope of the invention. (See, *e.g.*, Makrides, S., 1996, Microbiol. Revs 60:512-538 which is incorporated herein by reference in its entirety).

5.4.3 Increased Cytoplasmic Retention of Enzyme Substrates

The invention encompasses an embodiment where the marker gene product is an enzyme. This class of marker gene product is detected by the addition of its substrate attached to a labeled moiety. To successfully detect the tumor-targeted bacteria, the enzyme and substrate must be brought into close proximity. To aid in this process, the tumor-targeted bacteria can be modified to increase the retention of the enzyme substrate in its cytoplasm and to facilitate greater contact with the enzyme.

In a preferred mode of this embodiment, the enzyme is a pro-drug converting enzyme. This class of enzymes modulate the chemical nature of a benign drug to produce a cytotoxic agent and, as such, can also be used for treatment of a tumor as well as its detection and localization (see Section 5.6 and Table 2 below).

Enhanced cytoplasmic retention of the labeled enzyme substrate can be accomplished through the manipulation of the transport systems normally responsible for pumping the compounds out of the cell. Such transporters are called drug efflux systems.

One preferred modification of the host organism is to disable or severely compromise one or more drug efflux systems in the host organism. Membrane-associated energy driven efflux plays a major role in drug resistance in most organisms, including bacteria, yeasts, and mammalian cells (Nikaido, 1994, Science 264:382-388; Balzi *et al.*, 1994, Biochim Biophys Acta 1187:152-162; Gottesman *et al.*, 1993, Ann Rev Biochem 62:385). Normally, a wild type efflux system actively secretes potentially toxic compounds, thus reducing their accumulation inside the host organism.

In bacteria, a large number of efflux systems have been studied which can pump out a wide variety of structurally unrelated molecules ranging from, for example,

polyketide antibiotics (acrAE genes of *E. coli*, Ma *et al.* 1993, J Bacteriol 175:6299-6313), fluroquinolines and ethidium bromide (bmr of *Bacillus subtilis* and nor A of *Staphylococcus aureus*, Neyfakh *et al.* 1993, Antimicrob Agents Chemother 37:128-129), doxorubicin (drr of *Streptomyces peucetius*, to quaternary amines (qacE of *Klebsiella aerogenes* and mvrC of *E. coli*). See Table 1 for a list of non-limiting examples of efflux systems. Any such efflux systems may be used in a tumor-targeted vector of the invention.

The efflux systems that can be expressed in a tumor-targeting vector of the invention can also be of yeast or plant origin. Efflux systems in animals, plants, yeast, and bacteria are mechanistically related, often involving ATP binding cassette transporters (*see, e.g.*, Thomas *et al.*, 2000, Plant Cell 12(4):519-33). Recent data suggests that certain efflux systems are structurally conserved among yeast, plants and bacteria (Harley and Saier, 2000, J. Mol. Microbiol. Biotechnol.2(2):195-8). In one embodiment, a yeast gene which can be used as an efflux system is the bfr1+ gene, which confers brefeldin A resistance to *Schizosaccharomyces pombe* (Nagao *et al.*, 1995, J. Bacteriol. 177(6):1536-43). In another embodiment, a yeast gene which can be used as an efflux system is the CDR1 gene of *Candida albicans*, which confers resistance to cyclohexamide and chloramphenicol (Prasad *et al.*, 1995, Curr Genet 27:320-329).

Table 1. List of compounds that are secreted by active drug efflux systems

Chemical class	Specific name	Efflux systems
cationic dyes	rhomadamine-6G	bmr
	ethidium bromide	
	acriflavine	acrAE
basic antibiotics	puromycin	bmr
	doxorubicin	drr, mdr
hydrophilic antibiotics	novobiocin	acrAE
	macrolide	
hydrophobic antibiotics	beta-lactams	
organic cation	tetraphenyl phosphonium	bmr
uncharged	taxol	mdr
	chloramphenicol	bmr
weak acid	nalidixic acid	emr
	mithramycin	mdr
zwitterions	fluoroquinolines	bmr
detergent	SDS	acrAE

5.5. Useful Bacteria

Bacteria useful in the present invention are those facultative aerobic and facultative anaerobic bacteria which are able to differentiate between cancerous cells and non-cancerous counterpart cells. For example, the bacteria are able to differentiate between melanoma cells and melanocytes or between colon cancer cells and normal colon epithelial cells. Illustrative examples of bacteria useful in the present invention as tumor-targeted bacteria and/or for isolation of tumor-targeted bacteria include, but are not limited to the following facultative aerobes and anaerobes: *Escherichia coli*, including but not limited to pathogenic *Escherichia coli* (*e.g.*, entero-invasive or uropathogenic *Escherichia coli*), *Salmonella spp.*, *Shigella spp.*, *Streptococcus spp.*, *Yersinia enterocolitica*, *Listeria monocytogenies*, and *Mycoplasma hominis*. While, for ease of explanation, the description below refers specifically to *Salmonella*, the methods of and compositions used in the invention are in no way intended to be restricted to *Salmonella* but rather encompass any and all of the bacteria taught herein as useful.

Factors contributing to attenuation and tumor-targeting are described herein and may be used to construct or select an appropriate strain of bacteria for use in the methods of the invention. For example, methods to select and isolate tumor-targeted bacteria are described in Section 6.1, and methods to attenuate bacteria are described in Section 6.2 of International publication WO96/40238, which sections are incorporated herein by reference in their entirety. Examples of attenuated tumor-targeted bacteria are also described in International Application WO99/13053, which is incorporated herein by reference in its entirety.

Salmonella spp. are particularly useful strains for the present invention, since they show natural preference for attachment to and penetration into certain solid tumor cancer cells in tissue culture, as opposed to non-cancerous counterpart cells. (The term "*Salmonella*" is used generically herein to refer to any *Salmonella* species). Since *Salmonella* have a natural ability to distinguish between cancerous cells and their non-cancerous counterpart cells, and preferentially replicate in tumors (in mice), they are directly applicable to the methods for treatment according to the present invention.

Bacteria such as *Salmonella* are causative agents of disease in humans and animals. One such disease that can be caused by *Salmonella* is sepsis, which is a serious problem because of the high mortality rate associated with the onset of septic shock (Bone, 1993, Clinical Microbiol. Revs. 6:57-68). Therefore, to allow the safe use of *Salmonella* in the present invention, the bacterial vectors, including but not limited to *Salmonella*, are attenuated in their virulence for causing disease. In the present application, attenuation, in addition to its traditional definition in which a bacterium is modified so that

the bacterium is less pathogenic, is intended to include also the modification of a bacterial strain so that a lower titer of that derived bacterial strain can be administered to a patient and still achieve comparable results as if one had administered a higher titer of the parental bacterial strain. The end result serves to reduce the risk of toxic shock or other side effects due to administration of the strain to the patient. Such attenuated bacteria are isolated by means of a number of techniques. For example, attenuation can be achieved by the deletion or disruption of DNA sequences which encode for virulence factors that insure survival of the bacteria in the host cell, especially macrophages and neutrophils. Such deletion or disruption techniques are well known in the art and include, for example, homologous recombination, chemical mutagenesis, radiation mutagenesis, or transposon mutagenesis. Those virulence factors that are associated with survival in macrophages are usually specifically expressed within the macrophages in response to stress signals, for example, acidification, or in response to host cell defensive mechanisms such macropinocytosis (Fields *et al.*, 1986, Proc. Natl. Acad. Sci. USA 83:5189-5193). Table 4 of International Publication WO 96/40238 is an illustrative list of *Salmonella* virulence factors whose deletion results in attenuation.

Yet another method for the attenuation of the bacteria, such as *Salmonella*, is to modify substituents of the bacteria which are responsible for the toxicity of that bacteria. For example, lipopolysaccharide (LPS) or endotoxin is primarily responsible for the pathological effects of bacterial sepsis. The component of LPS which results in this response is lipid A ("LA"). Elimination or mitigation of the toxic effects of LA results in an attenuated bacteria since 1) the risk of septic shock in the patient is reduced and 2) higher levels of the bacterial strain can be tolerated.

Altering the LA content of bacteria, such as *Salmonella*, can be achieved by the introduction of mutations in the LPS biosynthetic pathway. Several enzymatic steps in LPS biosynthesis and the genetic loci controlling them in *Salmonella* have been identified (Raetz, 1993, J. Bacteriol. 175:5745-5753 and references therein), as well as corresponding mutants. One such illustrative mutant is *firA*, a mutation within the gene that encodes the enzyme UDP-3-O(R-30 hydroxymyristyl)-glycocyamine N-acyltransferase, which regulates the third step in endotoxin biosynthesis (Kelley *et al.*, 1993, J. Biol. Chem. 268:19866-19874). Bacterial strains bearing this type of mutation produce a lipid A that differs from wild-type lipid A in that it contains a seventh fatty acid, a hexadecanoic acid (Roy and Coleman, 1994, J. Bacteriol. 176:1639-1646). Roy and Coleman demonstrated that in addition to blocking the third step in endotoxin biosynthesis, the *firA*$^-$ mutation also decreases enzymatic activity of lipid A 4' kinase that regulates the sixth step of lipid A biosynthesis.

In addition to being attenuated, the bacteria of the invention are tumor-targeted, *i.e.*, the bacteria preferentially attaches to, infect, and/or remain viable in a tumor or tumor cell versus a normal tissue, non-tumor or non-tumor cell. Suitable methods for obtaining attenuated tumor-targeted bacteria are described in Sections 6.1 and 6.2 of International Publication WO 96/40238, which sections are incorporated by reference herein in their entirety. As the resulting bacteria are highly specific and, in one embodiment super-infective, the difference between the number of infecting bacteria found at the target tumor or tumor cell as compared to the non-cancerous counterparts becomes larger and larger as the dilution of the bacterial culture is increased such that lower titers of bacteria can be used with positive results. The techniques described in International Publication WO 96/40238 can also be used to produce attenuated tumor-targeted *Salmonella* or non-*Salmonella* bacterial strains.

An illustrative example of an attenuated tumor-targeted bacterium having an LPS pathway mutant is the *msbB*⁻ *Salmonella* mutant described in International Publication WO 99/13053, which is incorporated herein by reference in its entirety; see especially Section 6.1.2 which describes the characteristic of the *msbB- Salmonella* mutant. One characteristic of the *msbB*⁻ *Salmonella* is decreased ability to induce a TNF-α response compared to the wild-type bacterial strain. The *msbB*⁻ *Salmonella* induce TNF-α expression at levels of about 5 percent to about 40 percent compared to the levels induced by wild-type *Salmonella*.

The TNF-α response induced by whole bacteria or isolated or purified LPS can be assessed *in vitro* or *in vivo* using commercially available assay systems such as by enzyme linked immunoassay (ELISA). Comparison of TNF-α production on a per colony forming unit ("c.f.u.") or on a μg/kg basis, is used to determine relative activity. Lower TNF-α levels on a per unit basis indicate decreased induction of TNF-α production. In a preferred embodiment, the *msbB*⁻ *Salmonella* strain is modified to express a marker gene product which aids in reducing the volume of, or inhibiting the growth of, the solid tumor cancer.

The present invention also encompasses the use of derivatives of *msbB*⁻ attenuated tumor-targeted *Salmonella* mutants.

The stability of the attenuated phenotype is important such that the strain does not revert to a more virulent phenotype during the course of treatment of a patient. Such stability can be obtained, for example, by providing that the virulence gene is disrupted by deletion or other non-reverting mutations on the chromosomal level rather than epistatically.

Another method of insuring the attenuated phenotype is to engineer the

bacteria such that it is attenuated in more than one manner. For example, bacteria can be made much more susceptible to lysis by manipulating the cell wall integrity. In one embodiment, mutations or deletions in the *asd* locus are used to severely compromise cell wall integrity. This type of mutant bacteria relies on exogenously provided diaminopimelic acid to survive (Galan *et al.*, 1990, Gene 94:24-35). At the termination of therapy, this supplement can be withdrawn from the patient to accelerate the clearance of tumor-targeted bacteria. In other embodiments different pathways can be manipulated to attenuate the bacteria, such as, *e.g.*, a mutation in the pathway for lipid A production, such as the *msbB*⁻ mutation (International Publication WO 99/13053) and one or more mutations to auxotrophy for one or more nutrients or metabolites, such as uracil biosynthesis, purine biosynthesis, and arginine biosynthesis as described by Bochner, 1980, J. Bacteriol. 143:926-933. In a preferred embodiment, the tumor-targeted *msbB*⁻ *Salmonella* is also auxotrophic for purine. In certain embodiments, the attenuated tumor-targeted bacteria are attenuated by the presence of a mutation in *AroA, msbB, PurI* or *SerC*. In another embodiment the attenuated tumor-targeted bacteria are attenuated by the presence of a deletion in *AroA, msbB, PurI* or *SerC*.

Accordingly, any attenuated tumor-targeted bacteria may be used in the imaging and monitoring methods of the invention.

5.6. Vectors For Combining Treatment With Imaging

In certain embodiments of the present invention, the detection and monitoring methods can be combined with administration of a bacterial strain described in Section 5.5 which has been genetically modified to express one or more gene products of interest and serve as a bacterial vector which aids in reducing the volume of, or inhibiting the growth of, the solid tumor cancer. Hence, these embodiments encompass detection (and/or monitoring) and anti-tumor treatment, *i.e.*, inhibition of tumor growth or reduction of tumor volume. The same bacteria which express a marker gene can express an anti-tumor gene product. In specific modes of the embodiment, the marker gene itself is utilized as an anti-tumor gene. Alternatively, at least two different bacteria can be used, expressing a marker gene and one expressing an anti-tumor gene product (referred to herein as a "gene product of interest"). The gene product of interest is selected from the group consisting of proteinaceous and nucleic acid molecules. The nucleic acid molecule can be double-stranded or single-stranded DNA or double-stranded or single-stranded RNA, as well as triplex nucleic acid molecules. The nucleic acid molecule can function as a ribozyme, or antisense nucleic acid, *etc.* Release of the gene product of interest into the tumor environment may be enhanced in various ways. In one embodiment the gene product of

interest is co-expressed with a release factor (*e.g.*, BRP, see Section 5.4.1). In another embodiment, the gene product of interest is expressed as a fusion protein with an Omp-like protein, or portion thereof (see Section 5.4.2).

Antisense nucleotides are oligonucleotides that bind in a sequence-specific manner to nucleic acids, such as mRNA or DNA. When bound to mRNA that has complementary sequences, antisense prevents translation of the mRNA (see, *e.g.*, U.S. Patent Nos. 5,168,053; 5,190,931; 5,135,917; 5,087,617). Triplex molecules refer to single DNA strands that bind duplex DNA forming a collinear triplex molecule, thereby preventing transcription (see, *e.g.*, U.S. Patent No. 5,176,996).

A ribozyme is an RNA molecule that specifically cleaves RNA substrates, such as mRNA, resulting in inhibition or interference with cell growth or expression. There are at least five known classes of ribozymes involved in the cleavage and/or ligation of RNA chains. Ribozymes can be targeted to any RNA transcript and can catalytically cleave that transcript (see, *e.g.*, U.S. Patent No. 5,272,262; U.S. Patent No. 5,144,019; and U.S. Patent Nos. 5,168,053, 5,180,818, 5,116,742 and 5,093,246).

In various embodiments, the proteinaceous molecule is a cellular toxin, *e.g.*, saporin, a ribosome inactivating protein, or a porin protein, such as gonococcal PI porin protein. In other embodiments, the proteinaceous molecule is an anti-angiogenesis protein, an antibody or an antigen. In yet other embodiments, the proteinaceous molecule is a cytokine, *e.g.*, IL-2, or an anti-angiogenic factor, *e.g.*, endostatin, or a pro-drug converting enzyme, *e.g.*, Herpes Simplex Virus ("HSV") thymidine kinase or cytosine deaminase.

The nucleic acid molecule encoding the gene product of interest is from about 6 base pairs to about 100,000 base pairs in length. Preferably the nucleic acid molecule is from about 20 base pairs to about 50,000 base pairs in length. More preferably the nucleic acid molecule is from about 100 base pairs to about 10,000 base pairs in length. Even more preferably, it is a nucleic acid molecule from about 500 pairs to about 2,000 base pairs in length.

The nucleic acid encoding a gene product of interest is provided in an expression vector in operative linkage with a selected promoter, and optionally in operative linkage with other elements that participate in transcription, translation, localization, stability and the like.

In certain embodiments, the gene product of interest is cytotoxic or cytostatic to a cell by inhibiting cell growth through interference with protein synthesis or through disruption of the cell cycle. Such a product may act, for example, by cleaving rRNA or ribonucleoprotein, inhibiting an elongation factor, cleaving mRNA, or other mechanism that reduced protein synthesis to a level such that the cell cannot survive.

Examples of such cytotoxic or cytostatic molecules include but are not limited to saporin, the ricins, abrin, and other ribosome inactivating proteins (RIPs).

In another mode of this embodiment, the gene product of interest is a pro-drug converting enzyme or nucleic acid encoding the same, *i.e.* an enzyme that modulates the chemical nature of a drug to produce a cytotoxic agent. Illustrative examples of pro-drug converting enzymes include, HSV-TK, bacterial cytosine deaminase, cytochrome P-450 NADPH oxidoreductase, and those listed on page 33 and in Table 2 of WO 96/40238 by Pawelek *et al.*, which is incorporated herein in its entirety. Further examples of pro-drug converting enzymes can be seen in Table 2 below. WO 96/40238 also teaches methods for production of secreted fusion proteins comprising such pro-drug converting enzymes. According the present invention, a pro-drug converting enzyme need not be a secreted protein if co-expressed with a release factor such as BRP (see Section 5.4.1). In a specific embodiment, the pro-drug converting enzyme is cytochrome p450 NADPH oxidoreductase which acts upon mitomycin C and porfiromycin (Murray *et al.*, 1994, J. Pharmacol. Exp. Therapeut. 270:645-649).

In another mode of the embodiment, a gene product of interest is an inhibitor of inducible nitric oxide synthase (NOS) or of endothelial nitric oxide synthase. Nitric oxide (NO) is implicated to be involved in the regulation of vascular growth and in arteriosclerosis. NO is formed from L-arginine by nitric oxide synthase (NOS) and modulates immune, inflammatory and cardiovascular responses.

In another mode of the embodiment, the gene product of interest is cytotoxic or cytostatic to a cell by inhibiting the production or activity of a protein involved in cell proliferation, such as an oncogene or growth factor, (*e.g.*, bFGF, int-2, hst-1/K-FGF, FGF-5, hst-2/FGF-6, FGF-8) or cellular receptor or ligand. The inhibition can be at the level of transcription or translation (mediated by a gene product that is a ribozyme or triplex DNA), or at the level of protein activity (mediated by a gene product that is an inhibitor of a growth factor pathway, such as a dominant negative mutant).

In another mode of the embodiment, a gene product of interest is a cytokine, chemokine, or an immunomodulating protein or a nucleic acid encoding the same, such as interleukin-1 (IL-1), interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-10 (IL-10), interleukin-15 (IL-15), interleukin-18 (IL-18), endothelial monocyte activating protein-2 (EMAP2) GM-CSF, IFN-γ, IFN-α, MIP-3α, SLC, MIP-3β, or an MHC gene, such as HLA-B7. The cytokine may also be a member of the TNF family, including but not limited to, tumor necrosis factor-α (TNF-α), tumor necrosis factor-β (TNF-β), TNF-α-related apoptosis-inducing ligand (TRAIL), TNF-α-related activation-induced cytokine (TRANCE), TNF-α-related weak inducer of apoptosis (TWEAK), CD40 ligand (CD40L),

LT-α (lymphotoxin alpha), LT-β (lymphotoxin beta), OX40L (OX40 ligand), FasL, CD27L (CD27 ligand), CD30L (CD30 ligand), 4-1BBL, APRIL (a proliferation-inducing ligand), LIGHT (a 29 kDa type II transmembrane protein produced by activated T cells), TL1 (a tumor necrosis factor-like cytokine), TNFSF16, TNFSF17, and AITR-L (ligand of the activation-inducible TNFR family member). In other embodiment, the gene product of interest is tumor necrosis factor-α (TNF-α), tumor necrosis factor-β (TNF-β), TNF-α-related apoptosis-inducing ligand (TRAIL), TNF-α-related activation-induced cytokine (TRANCE), TNF-α-related weak inducer of apoptosis (TWEAK), and CD40 ligand (CD40L), or a functional fragment thereof.. For a review, see, *e.g.*, Kwon *et al.*, 1999, Curr. Opin. Immunol. 11:340-345, which describes members of the TNF family. Delivery of such immunomodulating these marker gene products will modulate the immune system, increasing the potential for host antitumor immunity. Alternatively, nucleic acid molecules encoding costimulatory molecules, such as B7.1 and B7.2, ligands for CD28 and CTLA-4 respectively, can also be delivered to enhance T cell mediated immunity. Yet another immunomodulating agent is, α-1,3-galactosyl transferase, whose expression on tumor cells allows complement-mediated cell killing. In yet another alternative mode of the embodiment, the immunomodulating agent is a tumor-associated antigen, *i.e.* a molecule specifically that is expressed by a tumor cell and not in the non-cancerous counterpart cell, or is expressed in the tumor cell at a higher level than in the non-cancerous counterpart cell. Illustrative examples of tumor-associated antigens are described in Kuby, 1992, *Immunology*, W.H. Freeman and Company, New York, NY, 1st Edition, pp. 515-520 which is incorporated by reference herein. Other examples of tumor-associated antigens are known to those of skill in the art.

In another mode of the embodiment, a gene product of interest is an anti-angiogenic molecule or protein associated with angiogenesis, such as, *e.g.*, endostatin, angiostatin, apomigren, anti-angiogenic antithrombin III, the 29 kDa N–terminal and a 40 kDa C-terminal proteolytic fragments of fibronectin, a uPA receptor antagonist, the 16 kDa proteolytic fragment of prolactin, the 7.8 kDa proteolytic fragment of platelet factor-4, the anti-angiogenic 24 amino acid fragment of platelet factor-4, the anti-angiogenic factor designated 13.40, the anti-angiogenic 22 amino acid peptide fragment of thrombospondin I, the anti-angiogenic 20 amino acid peptide fragment of SPARC, RGD and NGR containing peptides, the small anti-angiogenic peptides of laminin, fibronectin, procollagen and EGF, and peptide antagonists of integrin $\alpha_v\beta_3$ and the VEGF receptor. In a preferred embodiment of the invention, the anti-angiogenic molecule is a functional fragment of endostatin, apomigren or thrombospondin I.

In another mode of the embodiment, a gene product of interest is a Flt-3

ligand or nucleic acid encoding the same.

In another specific embodiment, the gene product of interest is a cytotoxic polypeptide or peptide, or a functional fragment thereof. Examples of cytotoxic polypeptides or peptides include, but are not limited to, members of the bacteriocin family (*see e.g.*, Konisky, 1982, Ann. Rev. Microbiol. 36:125-144), verotoxin, cytotoxic necrotic factor 1 (CNF1), cytotoxic necrotic factor 2 (CNF2), *Pasteurella multiocida* toxin (PMT), *Pseudomonas* endotoxin, hemolysin, CAAX tetrapeptides which are potent competitive inhibitors of farnesyltransferase, cyclin inhibitors, Raf kinase inhibitors, CDC kinase inhibitors, caspases, p53, p16, and p21. Examples of bacteriocin family members, include, but are not limited to, bacteriocin release protein (BRP), ColE1, ColE1a, ColE1b ColE2, ColE3, ColE4, ColE5, ColE6, ColE7, ColE8, ColE9, Colicins A, Colicin K, Colicin L, Colicin M, cloacin DF13, pesticin A1122, staphylococcin 1580, butyricin 7423, pyocin R1 or AP41, megacin A-216, and vibriocin. In a preferred embodiment, the gene product of interest is colicin E3. In another preferred embodiment, the gene product of interest is BRP.

For example, Colicin E3 (ColE3) has been shown to have a profoundly cytotoxic effect on mammalian cells (see, Smarda et al, 1978, Folia Microbiol. 23:272-277), including a leukemia cell model system (see, Fiska et al, 1978, Experimentia 35: 406-407). ColE3 cytotoxicity is a function of protein synthesis arrest, mediated by inhibition of 80S ribosomes (Turnowsky *et al.*, 1973, Biochem. Biophys. Res. Comm. 52:327-334). More specifically, ColE3 has ribonuclease activity (Saunders, 1978, Nature 274:113-114). In its naturally occurring form, ColE3 is a 60kDa protein complex consisting of a 50kDa and a 10kDa protein in a 1:1 ratio, the larger subunit having the nuclease activity and the smaller subunit having inhibitory function of the 50kDa subunit. Thus, the 50kDa protein acts as a cytotoxic protein (or toxin), and the 10kDa protein acts as an anti-toxin. Accordingly, in one embodiment, when ColE3 is used as a secondary marker gene product, the larger ColE3 subunit or an active fragment thereof is expressed alone or at higher levels than the smaller subunit. In a preferred mode of the embodiment, ColE3 expression is accompanied by BRP expression to enhance release into the tumor environment. In yet another embodiment of the invention, the ColE3 50kDa toxin and 10kDa anti-toxin are encoded on a single plasmid within an attenuated tumor-targeted bacteria, such as *Salmonella*. In this embodiment, the toxin/anti-toxin can act as a selection system for the *Salmonella* which carry the plasmid, such that *Salmonella* which lose the plasmid are killed by the toxin. In another embodiment, the 10kDa anti-toxin is on the chromosome, separate from the colE3 toxin on the plasmid, resulting in a barrier to transmission of other bacteria (see, Diaz *et al.*, 1994, Mol. Microbio. 13:855-861).

In another preferred mode of this embodiment, the bacteriocin is cloacin

DF13. Cloacin DF13 functions in an analogous manner to ColE3. The protein complex is of 67KDa molecular weight. The individual components are 57kDa and 9kDa in size. In addition to its ribonuclease activity, DF13 can cause the leakage of cellular potassium.

In yet another preferred mode of this embodiment, the bacteriocin is colicin V (*See, e.g.*, Pugsley, A.P. and Oudega, B. "Methods for Studing Colicins and their Plasmids" Plasmids a Practical Approach, 1987, ed. by K.G. Hardy; Gilson, L. *et al.*, 1990, The EMBO Journal vol. 9 pp3875-3884).

In another embodiment, the bacteriocin is selected from the group consisting of colicin E2 (a dual subunit colicin similar to ColE3 in structure but with endonuclease rather than ribonuclease activity); Colicins A, E1, Ia, Ib, or K, which form ion-permeable channels, causing a collapse of the proton motive force of the cell and leading to cell death; colicin L which inhibits protein, DNA & RNA synthesis; colicin M which causes cell sepsis by altering the osmotic environment of the cell; pesticin A1122 which functions in a manner similar to colicin B function; staphycoccin 1580, a pore-forming bacteriocin; butyricin 7423 which indirectly inhibits RNA, DNA and protein synthesis through an unknown target; Pyocin P1, or protein resembling a bacteriophage tail protein that kills cells by uncoupling respiration from solute transport; Pyocin AP41 which has a colicin E2-like mode of action; and megacin A-216 which is a phospholipase that causes leakage of intracellular material (for a general review of bacteriocins, see Konisky, 1982, Ann. Rev. Microbiol. 36:125-144).

In yet another mode of this embodiment, the bacteriocin is BRP. The cytotoxic activity of BRP is mediated by the release of cellular components.

In another specific embodiment, the gene products of interest are tumor inhibitory enzymes or functional fragments thereof. Examples of tumor inhibitory enzymes include, but are not limited to, methionase, asparaginase, lipase, phospholipase, protease, ribonuclease (excluding colE3), DNAse, and glycosidase. In a preferred embodiment, the primary marker gene product is methionase.

In a particular embodiment, the gene product of interest comprises a number of viral gene products. For example, the gene product of interest comprises all the viral proteins encoded by an adenovirus or herpes virus genome. In a particular example, the gene product of interest is all the viral proteins encoded by an adenovirus genome except for the E1B viral protein such that this particular adenovirus can only replicate in a mammalian cell lacking p53 activity.

Additional types of cellular toxins that may be delivered according to the methods of the present invention are antibody molecules that are preferably expressed within the target cell; hence, these antibody molecules have been given the name

"intrabodies." Conventional methods of antibody preparation and sequencing are useful in the preparation of intrabodies and the nucleic acid sequences encoding same; it is the site of action of intrabodies that confers particular novelty on such molecules. (For a review of various methods and compositions useful in the modulation of protein function in cells via the use of intrabodies, see International Application WO 96/07321).

Intrabodies are antibodies and antibody derivatives (including single-chain antibodies or "SCA") introduced into cells as transgenes that bind to and incapacitate an intracellular protein in the cell that expresses the intrabodies or derivatives. As used herein, intrabodies encompass monoclonals, single chain antibodies, V regions, and the like, as long as they bind to the target protein. Intrabodies to proteins involved in cell replication, tumorigenesis, and the like (*e.g.*, HER2/neu, VEGF, VEGF receptor, FGF receptor, FGF) are especially useful. The intrabody can also be a bispecific intrabody. Such a bispecific intrabody is engineered to recognize both (1) the desired epitope and (2) one of a variety of "trigger" molecules, *e.g.*, Fc receptors on myeloid cells, and CD3 and CD2 on T cells, that have been identified as being able to cause a cytotoxic T cell to destroy a particular target.

For example, antibodies to HER2/neu (also called erbB-2) may be used to inhibit the function of this protein. HER2/neu has a pivotal role in the progression of certain tumors, human breast, ovarian and non-small lung carcinoma. Thus, inhibiting the function of HER2/neu may result in slowing or halting tumor growth (see, *e.g.* U.S. Patent No. 5,587,458).

Nucleic acid molecules and oligonucleotides for use as described herein can be synthesized by any method known to those of skill in this art (see, *e.g.*, International Publication WO 93/01286, U.S. Patent Nos. 5,218,088; 5,175,269; 5,109,124). Identification of oligonucleotides and ribozymes for use as antisense agents and DNA encoding genes for targeted delivery for genetic therapy involve methods well known in the art. For example, the desirable properties, lengths and other characteristics of such oligonucleotides are well known. Antisense oligonucleotides may be designed to resist degradation by endogenous nucleolytic enzymes using linkages such as phosphorothioate, methylphosphonate, sulfone, sulfate, ketyl, phosphorodithioate, phosphoramidate, phosphate esters, and the like (*see, e.g.*, Stein in: *Oligodeoxynucleotides, Antisense Inhibitors of Gene Expression,* Cohen, Ed, Macmillan Press, London, pp. 97-117, 1989); Jager *et al.*, 1988, Biochemistry 27:7237).

Particularly useful antisense nucleotides and triplex molecules are molecules that are complementary to bind to the sense strand of DNA or mRNA that encodes a protein involved in cell proliferation, such as an oncogene or growth factor, (*e.g.*, bFGF, int-2, hst-1/K-FGF, FGF-5, hst-2/FGF-6, FGF-8). Other useful antisense oligonucleotides include

those that are specific for IL-8 (see, *e.g.*, U.S. Patent No. 5,241,049), c-*src*, c-*fos* H-*ras* (lung cancer), K-*ras* (breast cancer), urokinase (melanoma), BCL2 (T-cell lymphoma), IGF-1 (glioblastoma), IGF-1 (glioblastoma), IGF-1 receptor (glioblastoma), TGF-β1, and CRIPTO EGF receptor (colon cancer). These particular antisense plasmids reduce tumorigenicity in athymic and syngenic mice.

The nucleotide sequences of the genes encoding these gene products are well known (*see* GenBank). A nucleic acid molecule encoding one of the may be isolated by standard methods, such as amplification (*e.g.*, PCR), probe hybridization of genomic or cDNA libraries, antibody screening of expression libraries, chemically synthesized or obtained from commercial or other sources.

Nucleic acid molecules and oligonucleotides for use as described herein can be synthesized by any method known to those of skill in this art (see, *e.g.*, International Publication WO 93/01286, U.S. Patent Nos. 5,218,088; 5,175,269; 5,109,124). Identification of oligonucleotides and ribozymes for use as antisense agents involve methods well known in the art.

In certain embodiments, the gene product of interest is a fragment, analog, or variant of the wild-type full-length gene product, or a nucleic acid encoding the same. The derivative, analog or variant is functionally active, *e.g.*, capable of exhibiting one or more functional activities associated with a full-length, wild-type marker gene product. As one example, such derivatives, analogs or variants which have the desired therapeutic properties can be used to inhibit tumor growth. Derivatives or analogs of an marker gene product can be tested for the desired activity by procedures known in the art, including those described herein.

In particular, variants can be made by altering gene sequences by substitutions, additions (*e.g.*, insertions) or deletions that provide molecules having the same or increased anti-tumor function relative to the wild-type marker gene product. For example, the variants of the invention include, but are not limited to, those containing, as a primary amino acid sequence, all or part of the amino acid sequence of the gene product, including altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a silent change, *i.e.*, the altered sequence has at least one conservative substitution.

Any of the gene product of interest-encoding nucleic acids that are of mammalian origin can be altered to employ bacterial codon usage by methods known in the art. Preferred codon usage is exemplified in Current Protocols in Molecular Biology, Green Publishing Associates, Inc., and John Wiley & Sons, Inc. New York, and Zhang *et al.*, 1991, Gene 105: 61.

In certain embodiments, the gene product of interest is expressed as a fusion protein. In a specific embodiment, a gene product is constructed as a chimeric or fusion protein comprising the gene product or a fragment thereof joined at its amino- or carboxy-terminus via a peptide bond to an amino acid sequence of a different protein. In one mode of this embodiment, such a chimeric protein is produced by recombinant expression of a nucleic acid comprising or encoding the gene product of interest, *e.g.*, comprising a TNF encoding sequence, joined in-frame to a coding sequence for a different protein. Such a chimeric product can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other by methods known in the art, in the proper coding frame, and expressing the chimeric product into the expression vehicle of choice by methods commonly known in the art. Chimeric nucleic acids comprising portions of a nucleic acid comprising or encoding a gene product of interest fused to any heterologous protein-encoding sequence may be constructed. In a specific mode, the fusion protein comprises an affinity tag such as a hexahistidine tag, or other affinity tag that may be used in purification, isolation, identification, or assay of expression. In another specific mode of this embodiment, the fusion protein comprises a protease cleavage site such as a metal protease or serine cleavage site. In this particular mode, it is in some cases preferred that a protease site corresponding to a protease which is active at the site of a tumor is constructed into a fusion protein of the invention. In certain embodiments, an gene product of interest is constructed as a fusion protein to an Omp-like protein, or portion thereof (*e.g.*, signal sequence, leader sequence, periplasmic region, transmembrane domain, multiple transmembrane domains, or combinations thereof; see *supra*, Section 3.1 for definition of "Omp-like protein").

In another embodiment, the diagnostic imaging system, including but not limited to streptavidin, also has antitumor activity. In one embodiment, the diagnostic imaging system is combined with BRP, such as BRP and streptavidin, where the marker gene product streptavidin has increased antitumor activity when secreted. In another embodiment, the agent chelated by streptavidin has antitumor activity, such as would be mediated by high specific localization of gamma-emitting radioactive biotin. In another embodiment, the agent chelated by streptavidin has antitumor activity when activated by another agent, such as when boron is activated by neutrons (Sano, 1999, Bioconjug Chem. 10:905-911).

In another embodiment, the present invention provides methods for local delivery of one or more fusion proteins comprising a ferry peptide and a marker gene product to a solid tumor by tumor-targeted bacteria. Ferry peptides used in fusion proteins have been shown to facilitate the delivery of a polypeptide or peptide of interest to virtually any cell within diffusion limits of its production or introduction (see., *e.g.*, Bayley, 1999, Nature Biotechnology 17:1066-1067; Fernandez *et al.*, 1998, Nature Biotechnology 16:418-420; and Derossi *et al.*, 1998, Trends Cell Biol. 8:84-87). Accordingly, engineering tumor-targeted bacteria to express fusion proteins comprising a ferry peptide and a marker gene product enhances the ability of a marker gene product to be internalized by tumor cells. In a specific embodiment, tumor-targeted bacteria are engineered to express a nucleic acid molecule encoding a fusion protein comprising a ferry peptide and a marker gene product. In another embodiment, tumor-targeted bacteria are engineered to express one or more nucleic acid molecules encoding one or more fusion proteins comprising a ferry peptide and a marker gene product. Examples of ferry peptides include, but are not limited to, peptides derived from the HIV TAT protein, the antennapedia homeodomain (penetratin), Kaposi fibroblast growth factor (FGF) membrane-translocating sequence (MTS), and herpes simplex virus VP22.

The present invention also provides methods for local delivery of one or more fusion proteins comprising a signal peptide, ferry peptide and an marker gene product to a solid tumor by tumor-targeted bacteria. In a specific embodiment, tumor-targeted bacteria are engineered to express one or more nucleic acid molecules encoding one or more fusion proteins comprising a signal sequence, a ferry peptide and an marker gene product.

The present invention also provides methods for local delivery of one or more fusion proteins comprising a signal peptide, a proteolytic cleavage site, a ferry peptide and an marker gene product to a solid tumor by tumor-targeted bacteria. In a specific embodiment, tumor-targeted bacteria are engineered to express one or more nucleic acid molecules encoding one or more fusion proteins comprising a signal sequence, a proteolytic cleavage site, a ferry peptide and an marker gene product.

The present invention also provides methods for local delivery of one or more fusion proteins of the invention and one or more marker gene products of the invention to the site of a solid tumor by tumor-targeted bacteria. Preferably, the expression of both the fusion protein(s) and marker gene product(s) at the site of the solid tumor by an tumor-targeting bacteria improves the level of tumor or tumor cell growth inhibited compared to when either fusion protein(s) alone or the marker gene product(s) alone is expressed.

As discussed above, in certain embodiments of the invention, the marker

gene product can be a pro-drug converting enzyme or a pro-drug. The pro-drug converting enzyme can be fused or conjugated to a polypeptide therapeutic of the invention. Exemplary pro-drug converting enzymes are provided in Table 2 below.

TABLE 2

REPRESENTATIVE PRO-DRUG CONVERTING ENZYMES FOR USE IN VECTOR THERAPY

Enzyme	Pro-drug	Reference
Carboxypeptidase G2	benzoic acid mustards	Bashawe *et al.*, 1988; Springer *et al.*, 1990
	aniline mustards	Davies *et al.*, 1994
	phenol mustards	Springer *et al.*, 1990
Beta-glucuronidase	p-hydroxyaniline mustard-glucuronide	Roffer *et al.*, 1991
	epirubicin-glucuronide	Halsma *et al.*, 1992
		Mitaku *et al.*, 1994
Penicillin-V-amidase	adriamycin-N phenoxyaceryl	Kerr *et al.*, 1990
Penicillin-G-amidase	N-(4'-hydroxyphenyl acetyl)-palytoxin doxorubicin	Bignami *et al.*, 1992
	melphalan	Vrudhula *et al.*, 1993
β-lactamase	nitrogen mustard-cephalosporin	Alexander *et al.*, 1991
	β-phenylenediamine	
	vinblastine derivative-cephalosporin	
	cephalosporin mustard	Meyer *et al.*, 1993
		Svensson *et al.*, 1993
β-glucosidase	cyanophenylmethyl-β-D-gluco-pyranosiduronic acid	Rowlandson-Busza *et al.*, 1991
β-galactosidase	β-galactose-drug conjugates (*e.g.*, alkylating agents, purine and pyridmide antimetabolites,, anthracyclines, campothecin, and taxol)	Farquhar *et al.*, 1997
Nitroreductase	5-(adaridin-1-yl-)2, 4-dinitrobenzamide	Knox *et al.*, 1988; Somani and Wilman, 1994
Carboxylesterase	CPT-11 (Irinotecan)	Kojima *et al.*, 1998
Cytosine deaminase	5-FC (5-fluorocytosine)	Hamstra *et al.*, 1999;
Carboxypeptidase A	methotrexate-alanine	Haenseler *et al.*, 1992

Bagshawe *et al.*, 1988, Br. J. Cancer 58:700-703.
Springer *et al.*, 1990, J. Med. Chem. 33:677-681.

Davies *et al.*, 1994, Ann. Oncol. 5 (Suppl 5):73(abstr).

Springer *et al.*, 1994, A novel bisiodo-phenol mustard in antibody-directed enzyme pro-drug therapy (ADEPT). In: Programme of Eleventh Hammersmith Conference. Advances in the Application of Monoclonal Antibodies. London: Hammersmith Hospital (abstr).

Haisma *et al.*, 1992(a), Cancer Immunol. Immunother. 34:343-348.

Roffler *et al.*, 1991, Biochem. Pharmacol. 42:2062-2065.

Haisma *et al.*, 1992(b), Br. J. Cancer 88:474-478.

Mitaku *et al.*, 1994, Ann. Oncol. 5 (Suppl 5):76 (abstr).

Kerr *et al.*, 1990, Cancer Immunol. Immunother. 31:202-206.

Bignami *et al.*, 1992, Cancer Res. 52:5759-5764.

Vrudhula *et al.*, 1993, J. Med. Chem. 38:919-923.

Alexander *et al.*, 1991, Tetrahedron Lett. 32:3296-3272.

Meyer *et al.*, 1993, Cancer Res. 53:3956-3963.

Svensson *et al.*, 1992, Bioconj. Chem. 3:176-181.

Rowlandson-Busza *et al.*, 1991, Cytotoxicity following specific activation of amygladin. In: Monoclonal Antibodies, Epenetos AA (ed), London: Chapman & Hall, pp. 179-183.

Farquhar *et al.*, 1997, Proceedings of the American Association for Cancer Research 38, p. 613-14, abstract no. 4121.

Knox *et al.*, 1988, Biochem. Pharmacol. 41:4661-4669.

Somani *et al.*, 1994, Ann. Oncol. 5 (Suppl 5):73 (abstr).

Kojima *et al.*, 1998, J. Clin. Invest. 101(8):1789-96

Hamstra *et al.*, 1999, Hum. Gene Ther. 10(12):1993-2003

Haenseler, E., Esswein, A., Vitols, K.S., Montejano, V., Mueller *et al.*, 1992, Biochemistry 31:214-220.

As discussed above, in certain embodiments, imaging and treatment are combined in the same vector. In certain embodiments, the same recombinant gene expressed by the vector is both a marker gene and a therapeutic. In one non-limiting mode of the embodiment, the marker gene substrate is radiolabeled. For example, when the marker gene is TK, the substrate can be radiolabeled FIAU or GCV (*e.g.*, ^{131}I-FIAU or ^{131}I-GCV), the corresponding marker metabolite, by virtue of its radioactivity, will provide a therapeutic benefit on the patient as well as permitting tumor imaging. Similarly, wherein the marker gene encodes an antigen and a labeled antibody administered for imaging, the antibody can be radiolabeled and the radiolabel would serve both as an imaging tool and a therapeutic agent. The same principle can be applied to other embodiments, for example those in which a labeled compound is administered that is incorporated into the vector (such as labeled DAP in *asd* mutant vectors as described above), the labeled compound can be labeled with a radionuclide that is used for both imaging and therapy.

5.7. Promoters for Control of Both Marker and Therapeutic Genes

The marker gene product and/or the anti-tumor gene product must be expressed at the tumor site. Because the tumor can be in, but is not limited to, a mammalian or avian cell, the promoter controlling the expression of the marker gene product and/or the

anti-tumor gene product must be compatible with such cells. The mammalian cell can be, but is not limited to, human, canine, feline, equine, bovine, porcine, rodent, *etc.* The choice of promoter will depend on the type of target cell and the degree or type of expression control desired. Promoters that are suitable for use in the present invention include, but are not limited to, constitutive, inducible, tissue-specific, cell type-specific and temporal-specific and need not necessarily function in a mammalian cell. Another type of promoter useful in the present invention is an event-specific promoter which is active or up-regulated in response to the occurrence of an event, such as viral infection. For example, the HIV LTR is an event specific promoter. The promoter is inactive unless the *tat* gene product is present, which occurs upon HIV infection.

Exemplary promoters useful in the present invention include, but are not limited to, the SV40 early promoter region (Bernoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, *et al.*, 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner *et al.*, 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the cytomegalovirus ("CMV") promoter, the regulatory sequences of the tyrosinase gene which is active in melanoma cells (Siders *et al.*, 1998, Gen. Ther. 5:281-291), the regulatory sequences of the metallothionein gene (Brinster *et al.*, 1982, Nature 296:39-42); plant expression vectors comprising the nopaline synthetase promoter region (Herrera-Estrella *et al.*, Nature 303:209-213) or the cauliflower mosaic virus 35S RNA promoter (Gardner, *et al.*, 1981, Nucl. Acids Res. 9:2871), and the promoter of the photosynthetic enzyme ribulose biphosphate carboxylase (Herrera-Estrella *et al.*, 1984, Nature 310:115-120); promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter, and the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift *et al.*, 1984, Cell 38:639-646; Ornitz *et al.*, 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl *et al.*, 1984, Cell 38:647-658; Adames *et al.*, 1985, Nature 318:533-538; Alexander *et al.*, 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder *et al.*, 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert *et al.*, 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf *et al.*, 1985, Mol. Cell. Biol. 5:1639-1648; Hammer *et al.*, 1987, Science 235:53-58; alpha 1-

antitrypsin gene control region which is active in the liver (Kelsey *et al.*, 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram *et al.*, 1985, Nature 315:338-340; Kollias *et al.*, 1986, Cell 46:89-94; myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead *et al.*, 1987, Cell 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314:283-286), prostate specific antigen gene control region which is active in prostate cells, and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason *et al.*, 1986, Science 234:1372-1378).

Another exemplary promoter is one that has enhanced activity in the tumor environment; for example, a promoter that is activated by the anaerobic environment of the tumor such as the P1 promoter of the *pepT* gene. Activation of the P1 promoter is dependent on the FNR transcriptional activator (Strauch *et al.*, 1985, J. Bacteriol. 156:743-751). In a specific embodiment, the P1 promoter is a mutant promoter that is induced at higher levels under anaerobic conditions than the native P1 promoter, such as the pepT200 promoter whose activity in response to anaerobic conditions is induced by CRP-cAMP instead of FNR (Lombardo *et al.*, 1997, J. Bacteriol. 179:1909-1917). In another embodiment, an anaerobically-induced promoter is used, *e.g.*, the *potABCD* promoter. *potABCD* is an operon that is divergently expressed from *pepT* under anaerobic conditions. The promoter in the *pepT* gene responsible for this expression has been isolated (Lombardo *et al.*, 1997, J. Bacteriol. 179:1909-1917).

Yet another exemplary promoter is an antibiotic-induced promoter, such as the *tet* promoter of the Tn10 transposon. Alternatively, the promoter can be an antibiotic-induced promoter, such as the *tet* promoter of the Tn10 transposon. In a preferred embodiment, the *tet* promoter is a single-mer, which single-mer responds in an all-or-nothing manner to the presence of tetracycline and provides a genetically stable on-off switch. In another embodiment, the *tet* promoter is multimerized, for example three-fold. Such a multimer responds in a graded manner to the presence of tetracycline and provides a more manipulable system for control of gene expression. Promoter activity would then be induced by administering to a subject who has been treated with the attenuated tumor-targeted bacteria of the invention an appropriate dose of tetracycline. Although the *tet* inducible expression system was initially described for eukaryotic systems such as *Schizosaccharomyces pombe* (Faryar and Gatz, 1992, Current Genetics 21:345-349) and mammalian cells (Lang and Feingold, 1996, Gene 168:169-171), recent studies extend its applicability to bacterial cells. For example, Stieger *et al.* (1999, Gene 226:243-252) have shown 80-fold induction of the firefly luciferase gene upon tet induction when operably linked to the *tet* promoter. Optimization of the *tet* expression system for use according to

the methods of the invention is described in Section 6 below. An advantage of this promoter is that it is induced at very low levels of tetracycline, approximately 1/100th of the dosage required for antibiotic activity.

In addition to the promoter, repressor sequences, negative regulators, or tissue-specific silencers can be inserted to reduce non-specific expression of the gene product. Moreover, multiple repressor elements may be inserted in the promoter region. One type of repressor sequence is an insulator sequence. Illustrative examples of repressor sequences which silence background transcription are found in Dunaway *et al.*, 1997, Mol. Cell Biol. 17:182-129; Gdula *et al.*, 1996, Proc. Natl. Acad. Sci. USA 93:9378-9383; Chan *et al.*, 1996, J. Virol. 70:5312-5328. In certain embodiments, sequences which increase the expression of the gene product can be inserted in the expression vector, *e.g.*, ribosome binding sites. Expression levels of the transcript or translated product can be assayed by any method known in the art to ascertain which promoter/repressor sequences affect expression.

5.8. Methods And Compositions For Treatment

According to the present invention, a first tumor-targeted bacterial vector which expresses a first gene product is used for imaging or monitoring a tumor. Such first tumor-targeted bacterial vector for imaging or monitoring may be used alone or together with a second tumor-targeted bacterial vector which expresses a second gene product used to produce a tumor growth inhibitory response or a reduction of tumor volume in a subject (including but not limited to a human patient) having a solid tumor cancer. In one embodiment of the present invention, the method comprises administering to a subject, a pharmaceutical composition comprising an effective amount of facultative aerobic or facultative anaerobic tumor-targeted bacteria suitable for imaging or monitoring a tumor. In another embodiment of the present invention, the method comprises administering to a subject, a pharmaceutical composition comprising an effective amount of a first facultative aerobic or facultative anaerobic, attenuated, tumor-targeted bacteria suitable for imaging or monitoring a tumor and a second facultative aerobic or facultative anaerobic, attenuated, tumor-targeted bacteria suitable for producing a tumor growth inhibitory response or a reduction of tumor volume in the subject. In yet another embodiment, the method comprises administering, to a subject, a pharmaceutical composition comprising an effective amount of a facultative aerobic or facultative anaerobic, attenuated, tumor-targeted bacterial vector which has been genetically modified to express a gene product of interest, which aids in reducing the volume, or inhibiting the growth of the tumor, in combination with one or more a pharmaceutical compositions comprising an effective amount of

facultative aerobic or facultative anaerobic tumor-targeted bacteria suitable for imaging or monitoring a tumor. In a preferred embodiment, the method comprises administering, to a subject, a pharmaceutical composition comprising an effective amount of a facultative aerobic or facultative anaerobic, attenuated, tumor-targeted *Salmonella spp.* which expresses a gene product of interest which aids in reducing the volume or inhibiting the growth of a solid tumor together with a pharmaceutical composition comprising an effective amount of facultative aerobic or facultative anaerobic tumor-targeted bacteria suitable for imaging or monitoring a tumor.

Solid tumors include, but are not limited to, sarcomas, carcinomas or other solid tumor cancers, such as renal carcinoma, mesoendothelioma, bladder cancer, germ line tumors and tumors of the central nervous system, including, but not limited to, breast cancer, prostate cancer, cervical cancer, uterine cancer, lung cancer, ovarian cancer, testicular cancer, thyroid cancer, astrocytoma, glioma, pancreatic cancer, stomach cancer, liver cancer, colon cancer, and melanoma. The subject is preferably an animal, including but not limited to animals such as cows, pigs, chickens, *etc.*, and is preferably a mammal, and most preferably human. Effective treatment of a solid tumor, includes but is not limited to, inhibiting tumor growth, reducing tumor volume, *etc.*

The amount of the pharmaceutical composition of the invention which is effective in imaging or monitoring or the treatment of a solid tumor cancer will depend on the nature of the solid tumor, and can be determined by standard clinical techniques. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed will also depend on the route of administration, and the seriousness of the solid tumor, and should be decided according to the judgment of the practitioner and each patient's circumstances. However, suitable dosage ranges are generally from about 1.0 c.f.u./kg to about 1×10^{10} c.f.u./kg; optionally from about 1.0 c.f.u./kg to about 1×10^{9} c.f.u./kg; optionally from about 1.0 c.f.u./kg to about 1×10^{8} c.f.u./kg; optionally from about 1×10^{2} c.f.u./kg to about 1×10^{9} c.f.u./kg; optionally from about 1×10^{4} c.f.u./kg to about 1×10^{9} c.f.u./kg; optionally from about 1×10^{2} c.f.u./kg to about 1×10^{8} c.f.u./kg; optionally from about 1×10^{4} c.f.u./kg to about 1×10^{8} c.f.u./kg. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems.

Various delivery systems are known and can be used to administer a pharmaceutical composition of the present invention. Methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The compounds may be administered by any convenient route, for example by infusion or bolus injection, by absorption through

epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, *etc.*) and may be administered together with other biologically active agents. Administration can be systemic or local. In addition, it may be desirable to introduce the pharmaceutical compositions of the invention into the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir. Pulmonary administration can also be employed, *e.g.*, by use of an inhaler or nebulizer, and formulation with an aerosolizing agent.

In a specific embodiment, it may be desirable to administer the pharmaceutical compositions of the invention locally to the area in need of treatment; this may be achieved by, for example, and not by way of limitation, local infusion during surgery, by injection, by means of a catheter, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. In one embodiment, administration can be by direct injection at the site (or former site) of a malignant tumor or neoplastic or pre-neoplastic tissue.

In another embodiment, the bacteria or bacterial vector can be delivered in a controlled release system. In one embodiment, a pump may be used (*see* Langer, *supra*; Sefton, 1987, CRC Crit. Ref. Biomed. Eng. 14:201; Buchwald *et al.*, 1980, Surgery 88:507; Saudek *et al.*, 1989, N. Engl. J. Med. 321:574). In another embodiment, polymeric materials can be used (*see* Medical Applications of Controlled Release, 1974, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida; Controlled Drug Bioavailability, Drug Product Design and Performance, 1984, Smolen and Ball (eds.), Wiley, New York; Ranger and Peppas, 1983, J. Macromol. Sci. Rev. Macromol. Chem. 23:61; *see also* Levy *et al.*, 1985, Science 228:190; During *et al.*, 1989, Ann. Neurol. 25:351; Howard *et al.*, 1989, J. Neurosurg. 71:105). In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, *i.e.*, the brain, thus requiring only a fraction of the systemic dose (*see, e.g.*, Goodson, 1984, in Medical Applications of Controlled Release, *supra*, vol. 2, pp. 115-138).

Other controlled release systems are discussed in the review by Langer (1990, Science 249:1527-1533).

In a preferred embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lidocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit

dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

5.9. Considerations For Pharmaceutical Compositions

A composition of the invention should be administered in a carrier that is pharmaceutically acceptable. The term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia or receiving specific or individual approval from one or more generally recognized regulatory agencies for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Buffered saline is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion and the like. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin. Such compositions will contain a therapeutically effective amount of the Therapeutic, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration. In a preferred embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer.

The reported serious complications and deaths following administration of FIAU in patients raise concerns regarding the administration of FIAU in clinical trials, including radiolabeled imaging trials. It should be noted, however, that serious FIAU-related toxicities are dependent on total dose and length of administration. In the FIAU imaging methods, FIAU is given as a single dose and at doses considerably lower than that used in prior clinical trials (32 ng as compared to 1176 - 2940 mg). The calculations for the

actual dose (mg) of FIAU used in imaging are given below. The radiosynthesis of several 2'-fluoro nucleosides has been described including carrier-added and no carrier-added syntheses. The mode of synthesis, % yield, specific activity and the mass-dose equivalent is presented in Table 3.

Table 3. Radiosynthesis of 2'-fluoronucleosides

2'-fluoro-nucleoside	Study (ref. #)	Mode of Synthesis	Yield (%)	Specific Activity (GBq/mmol)	Dose (MBq)	Dose Equiv. (mg)
FIAU	[1]	[^{123}I], [^{131}I]*	~90%	110 - 600	148	0.092 - 0.49
	[2]	[^{131}I]*	-	100 - 200	148	0.28 - 0.56
	[1]	no carrier-added	~45%	-	148	0.000032

*carrier-added: 25 mg NaI

[1] Misra *et al.*, 1986, Appl. Radiat. Isot. 37:901-905.

[2] Tovell *et al.*, 1987, J. Med. Virol. 22:183-188.

The mass of radiopharmaceutical produced from a no carrier-added synthesis can be calculated using Avogadro's number, the half-life of the radionuclide (in sec), the molecular weight of the radiopharmaceutical and the administered dose (in Bq). The calculation of the mass-dose equivalent for a no carrier-added synthesis of [^{124}I]-FIAU is presented:

Avogadro's Number (Na)	=	6.023×10^{23} atoms/mole
[^{124}I] half-life ($t_{1/2}$)	=	3.46×10^{5} sec
[^{124}I] - FIAU dose (Bq)	=	148 MBq (4mCi)
[^{124}I] - FIAU molecular weight (MW)	=	376 g/mole
Number of radiolabeled molecules (Nm)	=	Bq (dps) x $t_{1/2}$ (sec)
	=	$(1.48 \times 10^{6}) \times (3.46 \times 10^{5})$
	=	5.12×10^{13} molecules
Moles	=	Nm/Na
	=	5.12×10^{13} molecules / 6.023×10^{23} molecules/mole
	=	8.50×10^{-11} moles
Mass (for a no carrier-added synthesis)	=	moles x MW
	=	8.50×10^{-11} moles x 376 g/mole
	=	3.2×10^{-8} g

= 32 ng

Review of all published phase 1 and phase 2 clinical studies involving FIAU administration, the published radiosynthesis procedures for labeling FIAU, and the calculation of the mass-dose equivalent for a no carrier-added synthesis of [^{124}I]-FIAU clearly demonstrates that the potential for toxicity associated with the projected diagnostic studies is very very low. For example, the equivalent mass for a 148 MBq (4 mCi) dose of no carrier-added [^{124}I]-FIAU proposed for PET imaging studies in patients is only 32 ng. A dose of 32 ng of FIAU represents only 1/110,000 to 1/3,720,000 of the daily dose (and $3.1x10^{-5}$ to $1.6x10^{-7}$ of the total dose) that was administered to patients in the 14 to 28 day hepatitis B clinical trials, where no FIAU-related toxicity was observed. Only in the 168 day clinical trial (H3X-PPPC) did severe liver, pancreatic and neurotoxicity appear.

Even assuming the synthesis results in a 1000-fold lower specific activity compared to a no carrier-added synthesis, this would amount to a pharmacologic dose of 32 μg of FIAU. A 32 μg dose of FIAU represents less than 0.03 to 1.0 percent of the daily dose that was administered to patients in the 14 to 28 day hepatitis B clinical trials, where no FIAU-related toxicity was observed.

5.10. Methods of Delivery

Various delivery systems are known and can be used to administer a pharmaceutical composition of the present invention. Methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, subcutaneous and intravenous. Administration can be systemic or local. In a specific embodiment, it may be desirable to administer the pharmaceutical compositions of the invention locally to the area in need of treatment; this may be achieved by, for example, and not by way of limitation, local infusion during surgery, by injection or by means of a catheter. In one embodiment, administration can be by direct injection at the site (or former site) of a tumor. The present invention, the tumor-targeted bacteria or bacterial vectors which express a gene product of interest and the pro-drug treatment, may be advantageously used in a combination method with one or more doses of irradiation to produce a tumor growth inhibitory response or a reduction of tumor volume, in a subject, including a human patient, having a solid tumor

cancer.

5.11. Kits

The invention also provides kits for carrying out the non-invasive detection methods of the invention and kits for carrying out the non-invasive detection and treatment methods of the invention. Such kits comprise in one or more containers a purified population of tumor-targeted bacteria and a labeled moiety (including but not limited to biotin, an antibody or a chemotactic peptide), substrate, or compound which is useful for the non-invasive imaging in conjunction with said population of tumor-targeted bacteria. The label can be a radionuclide, paramagnetic ion or fluorophore. In one embodiment, the label is sequestered.

In one embodiment, the tumor-targeted bacteria harbor a recombinant marker gene operably linked to a promoter. In a preferred mode of the embodiment, the marker gene is streptavidin. In another embodiment, the tumor-targeted bacteria is a mutant with an enhanced preference to incorporate a labeled compound. In a preferred mode of the embodiment bacteria is mutant for the *asd* locus, most preferably only a partial mutant for said locus.

In other embodiments, the kit further comprises a second population of tumor-targeted bacteria suitable for tumor therapy in the container in which the tumor-targeted bacteria useful for imaging is present or in a separate container. In yet other embodiments, the tumor-targeted bacteria suitable for imaging is also suitable for therapy.

In other embodiments, the kit further comprises a pharmaceutically acceptable carrier in the container in which the tumor-targeted bacteria is present or in a separate container.

Instructions are optionally included for using the bacteria to carry out the non-invasive imaging methods of the present invention.

Optionally associated with such kits can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

5.12. Exemplary Embodiment: Thymidine Kinase as a Marker Gene

As described above, the invention provides indirect methods of tumor imaging comprising administering to the subject a tumor-targeted bacterial vector containing a marker gene, wherein the tumor-targeted bacterial vector targets to the tumor and/or infects cells of the tumor, under conditions in which the marker gene is expressed in

the tumor-targeted bacterial vector, thereby generating a marker gene product; administering to the subject a labeled marker substrate under conditions in which the labeled marker substrate is metabolized by the marker gene product to produce a labeled marker metabolite which is substantially retained in the tumor-targeted bacterial vector throughout a time-period sufficient for imaging the labeled marker metabolite; and scanning the subject to detect the labeled marker metabolite, thereby imaging and detecting a tumor in the subject.

Merely for purposes of illustration and not limitation, the gene and a labeled marker substrate are selected as a self-complementary pair from the group consisting of: wild-type, natural mutant, or genetically-engineered herpes simplex virus-thymidine kinase or varicella zoster virus-thymidine kinase gene and a labeled nucleoside analog such as FIAU, ACV or GCV. In a particular mode of the embodiment, the nucleoside analog is a 2'-fluoro-nucleoside such as FIAU.

The labeled 2'-fluoro-nucleoside analogue can be 5-[^{123}I]-, 5-[^{124}I]- or 5-[^{131}I]-2'-fluoro-5-iodo-1-ß-D-arabinofuranosyl- uracil; 5-[^{18}F]-2'-fluoro-5-fluoro-1-ß-D-arabinofuranosyl- uracil; 2-[^{11}C]- or 5-([^{11}C]-methy)-2'-fluoro-5-methyl-1-ß-D-arabinofuranosyl- uracil; 2-[^{11}C]- or 5-([^{11}C]-ethyl)-2'-fluoro-5-ethyl-1-ß-D-arabinofuranosyl- uracil; 5-(2-[^{18}F]-ethyl)-2'-fluoro-5-(2- fluoro-ethyl)-1-ß-D-arabinofuranosyl- uracil; 5-[^{123}I]-, 5-[^{124}I]- or 5-[^{131}I]-2'-fluoro-5-iodovinyl-1ß-D-arabinofuranosyl- uracil; 5-[^{123}I]-, 5-[^{124}I]- or 5-[^{131}I]-2'- fluoro-5-iodo-1-ß-D-arabinofuranosyl- uracil; or 5-[^{123}I]-, 5-[^{124} I]- or 5-[^{131}I]-2'-fluoro-5-iodovinyl-1-ß-D-aribofuranosyl- uracil.

In certain embodiments, the TK can have a therapeutic as well as imaging purpose. Upon concurrent expression of the TK and administration of a labeled pro-drug (*e.g.* a labeled ganciclovir or acyclovir) to the subject, the pro-drug is phosphorylated in the periplasm of the microorganism which is freely permeable to nucleotide triphosphates. The labeled metabolites, phosphorylated ganciclovir or acycolvir, which are toxic false DNA precursors, readily pass out of the periplasm of the microorganism and into the cytoplasm and nucleus of the host cell. In addition to providing the signal for imaging, the phosphorylated ganciclovir or acycolvir incorporate into host cell DNA, thereby causing the death of the host cell.

In another embodiment, two recombinant tk genes are administered, either by way of two different bacterial vectors or a single bacteria vector comprising two recombinant tk genes. The two recombinant tk genes are selected for different substrate specificity and subcellular localization. For example, the imaging mutant tk gene is retained in the cytosol of a first tumor-targeted bacterial vector and the treatment mutant tk

gene is in the periplasm of a second tumor-targeted bacterial vector.

6. Examples

The following series of examples are presented by way of illustration and not by way of limitation on the scope of the invention. Instead, the examples are meant to illustrate certain variations of genetically engineered tumor-targeted *Salmonella* useful for the methods of the invention to detect, monitor and/or treat solid tumors.

6.1. Example: Tumor Detection Using Tumor-targeted *Salmonella* Expressing HSV1-TK

The initial study was conducted using s.c. implanted tumors to provide a standard easily reproduced tumor model. Mice (C57B/6) were subcutaneously implanted with C38 colon tumor cells and staged until the tumors were palpable (20 days). *Salmonella* expressing HSV1-TK from plasmid p5-3 (Pawelek *et al.*, 1997, Cancer Res. 57:4537-4544) in VNP20009 [strain YS1645 *purI*$^-$, *msbB*$^-$, *xyl*, *tet*^{s}, *amp*^{s}] (ATCC# 202165; WO 96/40238) were injected i.v. ($2x10^6$ cfu/animal) and after four days, 10 μCi [^{14}C]-FIAU was injected i.v. After 23 hours, the animals were sacrificed and muscle, liver and tumor tissue samples removed and homogenized for radioactivity (DPM) and bacterial cfu determinations per gram of tissue. The accumulation of [^{14}C]-FIAU is presented in Table 4.

Table 4.

Sample	mouse #	cfu/gram tissue	DPM/gram tissue
-bacteria			
muscle	1	-	474
muscle	2	-	949
liver	1	-	9800
liver	2	-	8394
tumor	1	-	9761
tumor	2	-	5818
+bacteria			
muscle	3	$5.0x10^3$	474
muscle	4	$4.6x10^3$	949
liver	3	$7.1x10^5$	9800
liver	4	$1.3x10^6$	8394

tumor	3	$1.2x10^9$	9761
tumor	4	$1.0x10^9$	5818

A sample of tumor and liver from mouse 3 were subjected to QAR. Cryosections were cut and exposed for quantitative analysis. The printouts of tumor and liver sections are shown in FIG. 1 and 2. In each of these figures, histology is on the left, digital autoradiogram is on the right and the merged image is in the center.

These figures clearly document the ability of *Salmonella* expressing HSV1-TK to specifically accumulate [^{14}C]-FIAU within the tumor, with little or no accumulation in the surrounding skin, and little or no relative accumulation in the liver. Hence the tumor-targeted *Salmonella* expressing HSV1-TK are useful when administered in combination with radiolabeled FIAU for tumor detection via *in vivo* imaging.

6.2. Example: Cytosolic HSV1-TK Expression

Expression of HSV1-TK in the *Salmonella* cytoplasm increases signal localization and detection over periplamic expression. A vector coding for cytosolic HSV1-TK is made with PCR primers [forward 5'-GATCCCATGGCTTCGTACCCCGGCC-3' (SEQ ID NO:1) and reverse 5'-CTAGAAGCTTCAGTGGCTATGGCAGGGC-3' (SEQ ID NO:2)] to generate a product. The template for the PCR reaction is a plasmid containing the HSV1-TK (McKnight, 1980, Nucleic Acids Research 8: 5949-5964). PCR is performed for example as 1 cycle of 95°C for 5 min: 35 cycles of 95°C for 1 min; 55°C for 1min; 72°C for 2 min: and 1 cycle of 72°C for 10 min using Ready-to-go PCR beads (Pharmacia) or equivalent. The approximately 1.5 kilobase PCR product is then gel purified, followed by restriction digestion with *Nco*I and *Hind*III and the product ligated into the *Nco*I and *Hind*III sites of pTrc99a (Pharmacia). TK activity is assayed using a modification of the method of Summers and Summers (1977, J. Virol. 24:314-318). The kinase substrates [^{125}IdC (Dupont/New England Nuclear) or ^{3}H-ganciclovir (Moravek Biochemicals)] are incubated with bacterial cell lysate at 37°C for 1 hour and then bound to DE81 paper (Whatman). After washing, the associated radioactivity is determined in a scintilation counter.

6.3. Example: Enhanced Specificity of HSV1-TK For FIAU

Using a combination of positive and negative selection (Black *et al.*, 1996, PNAS 9:3525-3529), active HSV1-tk mutants that are able to bind FIAU preferentially over thymidine can be selected. Six codons hypothesized as playing a role in substrate binding (Balasubramanian et. al., 1990, J. Gen. Virol. 71:2979-2987; Leu 159, Ile 160, Phe 161, Ala

168, Leu 169, and Leu 170) in wild type HSV1-tk are targeted for random mutagenesis. The construction of the random sequence inserts is essentially as described in Black *et al.* (1996, PNAS 9:3525-3529). Briefly, two random oligonucleotides are constructed: MB126 5'-TGGGAGCTCACATGCCCCGCCCCCGGCCCTCACCNNNNNNNNNGACCG CCATCCCATC-3' (SEQ ID NO:3) and MB127 5'-ATAAGGTACCGCGCGGCCGG GTAGCANNNNNNNNNGGCGATGGGATGGCGG-3' (SEQ ID NO:4) where N designates all 4 nucleotides in a 100% random equimolar mixture. The oligos are annealed, extended and amplified by PCR essentially as described by Black and Loeb (1993, Biochem. 32:11618-11626). The oligos are purified after synthesis, annealed and extended with Klenow fragment. The annealed oligos are then amplified by PCR using the primers: P1 : 5'-TGGGAGCTCACATGCCCCGCC-3' (SEQ ID NO:5) and P2: 5'-ATGAGGTACCG-3' (SEQ ID NO:6). This amplification generates the random inserts with the restriction sites *Sac*I and *Kpn*I.

In order to create the random library (Black and Hruby, 1990, J. Biol. Chem. 265:17584-17594; Black *et al.*, 1996, PNAS 9:3525-3529), the random sequence inserts from above are ligated into the wild type HSV1-tk open reading frame to replace the wild type segment. However, to avoid background activity due simply to uncut wild type HSV1-tk vector, a dummy vector is created. This vector (pET23d backbone for high overexpression in the BL21DE3 strain) contains the wild type HSV1-tk open reading frame with a piece of non-functional DNA at the insertion site. This vector has no HSV1-TK activity unless there is successful replacement of the dummy fragment with a fragment from the random inserts that results in the production of an active enzyme. The dummy and PCR amplified random inserts are cut with *Kpn*I and *Sac* I and gel isolated. The vector and gel purified PCR amplified random inserts are ligated with *Kpn*I and *Sac*I. *E. coli* strain BL21(DE3) tdk$^-$ [F$^-$ *ompT hsdSB*(rB$^-$ mB$^-$)*gal dcm tdk* (DE3)] (Black *et al.*, 1996, PNAS 9:3525-3529) is transformed with the ligated mixture. The endogenous HSV1-tk in this strain has been deleted so only those bacteria that receive a HSV1-tk construct that is active will survive the initial positive selection screen.

In order to create a useful HSV1-TK that is able to bind FIAU with a high affinity, the enzyme expressed must be an active enzyme. Any clone harboring a HSV1-tk construct that does not yield active HSV1-TK enzyme is eliminated. In order to accomplish this, the first selection of the library is based on HSV1-TK activity, by the method of Black and Loeb (1993, Biochem. 32:11618-11626). After transformation and recovery, the transformants are plated onto LB + carbenicillin (carb ^{50}) to score total transformants. Individual colonies are then picked and streaked onto HSV1-TK selection media (2% BBL Trypticase peptone, 0.5% NaCl, 0.8% Gel-Rite, 0.2% glucose, 50mg/mL carb, 10mg/mL 5'-

fluorodeoxyuridine (FUdR), 2mg/mL thymidine, 12.5mg/mL uridine) and LB+carb ^{50} in parallel. The basis of the selection is that FUdR is phosphorylated by HSV1-TK to form FdUMP which is an inhibitor of thymidiylate synthase and thus the inhibition of dTMP production. The requirement for dTMP can therefore only be filled by an active HSV1-TK enzyme. Since the endogenous HSV1-TK enzyme in strain KY895 has been disrupted, the only active HSV1-TK must come from the random insert library. Only those clones that have received an active form of HSV1-TK will be able to grow on this selection media. Uridine is added to inhibit the thymidine phosphatase. HSV1-tk $^{+}$ clones are scored after incubation at 37°C for 24 hours.

Selection of FIAU sensitive clones is performed essentially as in the method of Black *et al.* (1996, PNAS 9:3525-3529) with minor modifications. All positive clones from above are picked and inoculated into HSV1-TK selection media in a 96 well plate. All clones are serial diluted into saline and streaked onto HSV1-TK selection plates that contain 2mg/ml thymidine or HSV1-TK selection plates that contain 1mg/ml of thymidine plus decreasing concentrations of FIAU (2mg/ml to 0mg/ml). Those clones that are able to bind and thus phosphorylate FIAU over thymidine are not able to grow on plates containing FIAU, since the HSV1-TK enzyme is bound to the FIAU and thus not available to phosphorylate the thymidine needed for cell survival.

6.4. Example: Enhanced Activation of Ganciclovir by HSV1-TK

This example illustrates a genetically engineered *Salmonella* expressing a mutant TK enzyme having an enhanced binding affinity for a pro-drug such as ganciclovir. Such *Salmonella* are useful in one embodiment of the invention, *i.e.*, in a method to detect and treat a solid tumor. The enhanced-ganciclovir-affinity TK advantageously provides a therapeutic effect and is used in combination with *Salmonella* expressing TK having an affinity for radiolabeled FIAU which permits *in vivo* detection of a solid tumor upon administration of the bacteria.

Salmonella HSV1-TK, where the TK enzyme is mutant and capable of binding ganciclovir at a higher affinity than wild type, is generated according the method published by Black *et al.*(1996, PNAS 9:3525-3529). These authors demonstrated the isolation of an HSV1-TK mutant with a 40-fold increase in specificity for ganciclovir. When this gene was expressed in tumor cells, a dramatic increase in sensitivity to ganciclovir was observed. The protein sequence of the naturally occurring form and the mutant are shown:

HSV1-TK ^{159}LIFDRHPIAALLCYP^{173} (SEQ ID NO:7)

Mutant #75 ^{159}LLLDRHPIACMLCYP^{173} (SEQ ID NO:8)

The mutant sequence is introduced into the coding sequence of HSV1-tk by PCR-mediated site directed mutagenesis (Ausubel *et al.*, 1995, in Short Protocols in Molecular Biology, third edition, NY:18.6-18.22). The mutations are introduced using two paired sets of primers, each of which generates approximately half of the complete sequence with a slight overlap and can be spliced together using a shared *Sau*3AI site engineered to occur in the overlap: Set 1 PCR primers (forward) 5'-GATCCCATGGCTTCGTACCCCGGCC-3' (SEQ ID NO:9) and (reverse) 5'-CGGCGATCGG ATGGCGGTCGAGGAGGAGGG-3' (SEQ ID NO:10); Set 2 PCR primers (forward) 5'- CCATCCGATC GCCGTCATGC TGTGC-3' (SEQ ID NO:11) and (reverse) 5'-CTAGAAGCTTCAGTGGCTATGGCAGGGC-3' (SEQ ID NO:12) are used to generate a product using wild type tk as a template, followed by restriction digestion with 1) PCR set 1, *Nco*I and *Sau*3AI, and 2) PCR set 2, *Sau*3AI and *Hind*III, and the two products simultaneously ligated into the *Nco*I and *Hind*III sites of pTrc99a (Pharmacia). Clones derived are tested for ganciclovir and FIAU activation profiles using the HSV1-TK enzyme assay based on Summers and Summers (1977, J. Virol. 24:314-318) and described above.

The HSV1-tk mutant described above (mutant #75; Black *et al.*, 1996, PNAS 9:3525-3529) is a "super" mutant in that it has a higher affinity for ganciclovir and a lower affinity for thymidine than wild type HSV1-TK. Because this enzyme has these properties it is more toxic to cells primarily because it is less able to bind thymidine so there is less competition in the cells for thymidine and thus more enzyme available to bind and metabolize the ganciclovir pro-drug.

6.5. Example: Optimal Time For Imaging After Labeled FIAU Intravenous Administration

It has been repeatedly observed that *Salmonella* targeting of tumors progresses over the first 3 to 5 days, with day 5 exhibiting the full targeting potential in all cases (Pawelek *et al.*, 1997, Cancer Res. 57:4537-454; Zheng *et al.*, 1997, Annual Meeting of the American Association for Cancer Research, abstract 60; King *et al.*, 1998, Annual Meeting of the American Association for Cancer Research, abstract 3484; Low *et al.*, 1999, Nature Biotech. 17:37-41). It is advantageous to determine the optimal time for imaging after radiolabeled FIAU administration in order to achieve a maximum "signal to background".

The optimal imaging times for *in vivo* tumor detection can be determined as described below.

Mice (C57B/6) are implanted with C38 colon tumor cells and staged until

the tumors are palpable. *Salmonella* expressing HSV1-TK or HSV1-"super" TK are injected into the tumor and after five days [^{131}I]- FIAU is injected intravenously. Gamma camera imaging is performed at various times, for example1 hr, 3 hr, 9 hr, 24 hr and 48 hr after FIAU administration. The accumulation of radiolabeled FIAU present in tumors and selected organs can also be determined in animals sacrificed for example at 24 and 48 hours post FIAU administration. Tissue is processed for determination of radioactive FIAU content incorporated into DNA by acid precipitation. Total tissue radioactivity is determined and the percent acid precipitated (% incorporated into DNA) versus the percent acid soluble (% as unincorporated FIAU, % unincorporated phosphorylated FIAU, and % radiolabeled metabolites) is determined. Tissue radioactivity (% dose/gram) is plotted versus time after FIAU injection.

6.6. Example: Time Course of Bacterial And Radioactivity Localization After Direct Intra-tumoral Administration of *Salmonella*

Salmonella have been demonstrated to target a wide variety of solid tumors following systemic administration. However, some tumor types, including glioma and prostate cancers may benefit from local-regional administration. The utility of tumor-targeted genetically engineered HSV1-TK expressing *Salmonella* injected either i.v. or by direct intra-tumoral administration for detection, monitoring and/or therapy can be assessed. Although i.v. administration of genetically altered *Salmonella* with selective "biological" targeting of the tumor *in vivo* is a preferred mode of application, it is clinically feasible to directly administer these bacteria into the tumor bed at time of surgery.

Mice (C57B/6) are implanted with C38 colon tumor cells and staged until the tumors are palpable. *Salmonella* expressing HSV1-TK or HSV1-"super" TK is injected directly into the tumor when tumors are palpable. [^{123}I]-FIAU is administered 1, 2, 4, 6, 10 or 14 days after *Salmonella* injection. Gamma camera imaging is done at 1 hr, 3 hr, 9 hr, 24 hr and 48 hr after FIAU administration. The accumulation of radiolabeled FIAU present in tumors and selected organs is also determined in animals sacrificed at 24 and 48 hours. Tissue is processed for determination of radioactive FIAU content incorporated into DNA by acid precipitation. Total tissue radioactivity is determined and the percent acid precipitated (% incorporated into DNA) versus the percent acid soluble (% as unincorporated FIAU, % unincorporated phosphorylated FIAU, and % radiolabeled metabolites) is determined. Data is plotted as tissue radioactivity (% dose/gram) versus time after *Salmonella* administration, bacterial count (number of bacteria/gram) versus time after *Salmonella* administration and bacterial count/tissue radioactivity ratio (number of bacteria/% dose) vs. time after *Salmonella* administration.

6.7. Other Tumor Models

The use of tumor-targeted genetically engineered *Salmonella* for imaging can be assessed in a spectrum of solid tumors and tumor growth environments. Orthotopic models include liver tumors arising from murine C38 colon carcinoma, lung tumors arising from B16F10 melanoma and breast tumors arising either from MDA-MB-231 or spontaneously in C-neu transgenic mice. Bacteria targeting and imaging are compared in orthotopically placed tumors (liver, lung and breast) with s.c. tumors as a reference. The spontaneous breast tumor model also provides metastases to multiple sites for assay in this system. *Salmonella* bacteria expressing a labeled marker gene are administered intravenously in combination, *e.g.*, simultaneously or separately within a specific time period, with labeled marker substrate and tumor detection follows the protocol described above. The animal is scanned and images of tumor are obtained. This imaging/treatment technique is broadly applicable to different orthotopic tumor sites and different tumor cell lines.

6.8. Example: Detection of Tumor-specific Bacteria Mediated by Biotin Binding

The gene containing streptavidin was obtained from the plasmid BBG9 R & D Systems, Minneapolis, MN). The plasmid was first transformed to *Salmonella* strain YS501 and re analyzed for restriction digestion pattern, and then subsequently transformed to the tumor specific strain YS1646 with and without the Bacteriocin Release Protein (BRP) plasmid pSW1 (Bio 101, Vista, CA). Streptavidin production in the supernatant was detected by inducing a streptavidin/BRP-containing strain with 1.0 μg/ml mitomycin for 4 hrs followed by centrifugation. Supernatant samples were spotted to nitrocellulose to allow binding, followed by a blocking step containing 3% bovine serum albumin and 0.05% Tween 20 in phosphate buffered saline. The presence of streptavidin was detected by overlaying the nitrocellulose strip with a biotin-conjugated alkaline phosphatase (Sigma, St. Louis, MO), followed by washing and then a color detection using nitro blue tetrazolium and 5-bromo-1-chloro-3-indol phosphate (NBT/BCIP). By comparison with a serial dilution of a streptavidin standard using the same assay, the tumor-specific bacterial supernatant was determined to contain 1 μg/ml or greater.

6.9. Example: Detection of Tumor-specific Bacteria Mediated Fluorescence

Green fluorescent protein has been used to demonstrate the localization of tumors (Yang *et al.*, 2000, Whole-body optical imaging of green fluorescent protein-expressing tumors and metastases. Proc Natl Acad Sci U S A. 97:1206-11), however, efficient means of delivery of GFP by systemically administered vectors has not been

shown.

Green fluorescence protein (GFP) was obtained from Clonetech (Palo Alto, CA) and subcloned into the ptrc99a expression vector (Pharmacia, Piscataway, NJ). The resulting trc-GFP plasmid was transformed into tumor-specific *Salmonella* strain VNP20009. GFP-containing *Salmonella* strain VNP20009 were injected i.v. (2×10^6) into mice bearing HTB117 human lung carcinoma. After 5 days, the mice were sacrificed and tumors were examined for the presence of fluorescent bacteria using standard cryomicrotomy and fluorescent microscopy techniques (FIG. 3). Figure 3A shows fluorescence of an untreated tumor and figure 3B shows fluorescence observed in tumors following treatment with GFP-containing *Salmonella* strain VNP20009.

These results demonstrate that a tumor specific bacterium can express GFP, and that GFP can be localized to tumors using tumor-specific bacteria.

6.10. Example: Cloning β-galactosidase Into Tumor-specific *Salmonella*

The LacZ open reading frame was cloned into the expression vector trc99a (Pharmacia) as a BspHI/StyI fragment from a promoterless β-gal vector (Genbank Accession #AF192277) and transformed into *Salmonella* YS1646. Positive clones were detected as blue colonies on X-gal plates.

LacZY was mobilized into *Salmonella* YS1646 as a F' factor containing the entire lacZ operon, including the lacY gene, which is responsible for lactose transport, as follows: *Salmonella* strain YS1646 was engineered to express the F' as follows such that the strain is able to be infected by phage. *Salmonella* strain YS501 (*recD*$^-$, chloramphenicol resistant) was mated with *E. coli* strain NH4104, which carries the F' plasmid containing the lactose operon, and *Salmonella* colonies were selected for chloramphenicol resistance, lac$^+$ on minimal media containing lactose and chloramphenicol. This strain, designated YS501-F' (which is also *met*$^-$) was mated with *Salmonella* strain YS1646 (which is also *pur*$^-$) and *Salmonella* colonies were selected on minimal media containing lactose and purine but lacking methionine. This strain was designated YS1646-F'. The presence of the F' lac results in lac+ *Salmonella*.

6.11. Example: Cloning Firefly Luciferase Into Tumor-specific *Salmonella*

The plasmid pSPLuc+ containing the gene for firefly luciferase was obtained from Promega (Madison, WI). pSPLuc+ was digested with NcoI and XbaI and ligated into pTrc99a (Pharmacia, NJ). This clone was then amplified by PCR using primers forward 5'-GTGTGCGGCCGCAATATTACTGAAATGAGCTGTTGACAATTAATCATCC-3' (SEQ ID NO:13) and reverse 5'-

GTGTGATATCGCATGCCTGCAGGTCGACTCTAGAATTAC-3' (SEQ ID NO:14) which add NotI and EcoRV sites to the ends of the luciferase open reading frame. The resulting product was subjected to restriction digestion with NotI and EcoRV and cloned into a promoterless β-galactosidase vector (Genbank # AF192277) also cut with NotI and EcoRV to remove most of the β-galactosidase coding region. The plasmid was transferred to VNP20009. The resulting clone has the partially constitutive trc promoter driving expression of the luciferase gene expression and results in functional expression of the luciferase protein. This demonstrates that tumor-specific bacteria can express luciferase.

6.12. Example: Non-*Salmonella* Bacteria Target Tumors and Inhibit Tumor Growth

6.12.1 Materials and Methods

Shigella flexneri ("*Shigella*"), a gram-negative *Shigella* species which functions under both aerobic and anaerobic conditions, and *Listeria monocytogenes* ("*Listeria*"; ATCC strain 43251), a gram-positive species which functions under aerobic and anaerobic conditions, were grown at 37°C either in Luria broth (LB) liquid media with shaking at approximately 225 revolutions per minute, or on LB solid media containing 1.5% agar. LB consisted of 10 g tryptone, 5 g yeast extract and 10 g NaCl per liter. The pH of LB was adjusted to 7 using a 1N solution of NaOH. Generally, cultures were streaked out on solid media and incubated until visible growth was observed, and then introduced into liquid media and grown to an appropriate density before freezing in 15% glycerol at -80°C. *Shigella* was grown to an OD_{600} of 1.9, corresponding to 1.03×10^9 c.f.u./ml. *Listeria* was grown to an OD_{600} of 0.6, corresponding to 0.98×10^8 c.f.u./ml.

Streptococcus agalactiae ("*Streptococcus*"; ATCC strain 13813), a *Streptococcus* species which functions under both aerobic and anaerobic conditions, was grown in heart brain infusion ("BHI"; Difco) liquid media or on BHI solid media according to the methods described for *Shigella* and *Listeria*. *Streptococcus* was grown to an OD600 of 0.85, corresponding to an estimated 2.5×10^8 c.f.u./ml. The bacterial culture was used without freezing for tumor-targeting experiments (Section 6.12.2 below). For tumor inhibition experiments (Section 6.12.3 below), *Streptococcus* colonies were inoculated into liquid culture as described above, grown to an OD600 of 1.2, and frozen at -80°C in 15% glycerol, corresponding to 1.2×10^8 c.f.u./ml after thawing.

6.12.2 Non-*Salmonella* Genera Target Selectively to Solid Tumors

To determine the tumor-targeting capabilities of *Shigella*, *Listeria* and *Streptococcus*, the bacteria were administered to mice having established melanomas, and the ratio of the concentration of bacteria in the tumor to the concentration of bacteria in the

liver in each mouse was determined as follows.

The mice were implanted with B16F10 murine melanoma tumor cells and the bacteria administered when the tumors weighed approximately 1g, or approximately after 14-16 days post tumor cell implantation. Prior to administration, frozen *Shigella* and *Listeria* cultures were thawed at room temperature and diluted into phosphate buffered saline (PBS). For *Streptococcus* administration, a fresh culture was diluted into PBS. The bacteria were administered intravenously into the mice in the following amounts: 2×10^6 c.f.u. of *Shigella*; 1×10^4 or 1×10^5 c.f.u. of *Listeria*; and 1×10^5 or 1×10^6 c.f.u. of *Streptococcus*.

At day 5 following administration of the bacteria, the mice were sacrificed and tumors and livers harvested and homogenized. Serial dilutions of the homogenates were then plated to the appropriate media for each species.

The results of these experiments are shown in Table 5. These data indicate that *Shigella*, *Listeria*, and *Streptococcus*, which function under both aerobic and anaerobic conditions, have highly significant targeting ratios for tumors as compared to normal tissues (liver) when administered *in vivo*.

Table 5.

Strain Dose	Tumor c.f.u./g (mean ± SE)	Liver c.f.u./ (mean ± SE)	Ratio	Strain
2×10^6	$6.7 \pm 5.8 \times 10^7$	$5.5 \pm 5.8 \times 10^2$	120,000:1	*Shigella*
1×10^4	$2.7 \pm 2.4 \times 10^8$	$7.3 \pm 6.9 \times 10^5$	370:1	*Listeria*
1×10^5	$2.3 \pm 0.8 \times 10^8$	$3.1 \pm 1.9 \times 10^6$	70:1	*Listeria*
1×10^5	$6.1 \pm 2.9 \times 10^8$	$2.4 \pm 2.4 \times 10^3$	250,000:1	*Streptococcus*
1×10^6	$3.0 \pm 1.6 \times 10^9$	$2.5 \pm 2.5 \times 10^2$	12,000,000:1	*Streptococcus*

Tumor to normal tissue (liver) relative accumulations. Counts are based on colony forming units (c.f.u.) given as the mean ± standard error (SE).

6.12.3 Administration of *Non-Salmonella* Genera Reduces Volume or Inhibits Growth of Solid Tumors

To determine the ability of the tumor-targeting *Shigella*, *Listeria* and *Streptococcus* to inhibit tumor growth, the effect of administration of the tumor-specific bacteria on the growth of established melanomas was determined as follows:

Mice were implanted subcutaneously with B16F10 murine melanoma tumor cells (5×10^5 cells per animal). The *Shigella*, *Listeria* or *Streptococcus* were administered intravenously when the tumors weighed approximately 0.3 g.. Frozen stock of the bacteria

(see Section 6.12.1) were thawed at room temperature and diluted into PBS. The bacteria were administered intravenously into the mice in the following amounts: 2 x 10^6 c.f.u. of *Shigella*; 1 x 10^5 c.f.u. of *Listeria*; and 1 x 10^6 c.f.u. of *Streptococcus*.

Following the first week of administration of the tumor-specific bacteria, two doses (one on day 5, one on day 8 post-bacteria) of 500 mg ampicillin/kg body weight were administered to the mice that had received *Listeria*, for the alleviation of systemic effects of the bacteria.

Tumor volume was monitored approximately every 5 days. Tumor growth is graphically depicted as tumor volume versus time in FIG. 4. Tumor growth in mice receiving the tumor-targeted *Listeria*, *Shigella* or *Streptococcus* was inhibited by at least approximately 40% relative to tumor growth in control animals, and up to approximately 65% in the case of *Listeria*. These data indicate that these facultative, gram positive and gram negative bacteria reduce tumor volume or inhibit tumor growth.

7. Microorganism Deposits

As described in WO99/13053 published March 18, 1999, the following microorganisms were deposited with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, Virginia 20110-2209, on 25 August, 1998, and have been assigned the indicated Accession numbers:

Microorganism	ATCC Accession No.
YS1646	202165
YS1456	202164

The invention claimed and described herein is not to be limited in scope by the specific embodiments, including but not limited to the deposited microorganism embodiments, herein disclosed since these embodiments are intended as illustrations of several aspects of the invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

A number of references are cited herein, the entire disclosures of which are incorporated herein, in their entirety, by reference.

We claim:

1. A method of imaging a tumor in a subject comprising:

(a) administering to the subject a tumor-targeted bacteria containing a marker gene, wherein the tumor-targeted bacteria targets the tumor cells and/or the tumor environment and the marker gene is expressed in the tumor-targeted bacteria, thereby generating a marker gene product; and

(b) scanning the subject to detect the marker gene product, thereby imaging the tumor in the subject.

2. The method of claim 1, wherein the marker gene is detected directly.

3. The method of claim 2, wherein the marker gene encodes a fluorescent protein.

4. The method of claim 3, wherein the fluorescent protein is green fluorescent protein, blue fluorescent protein, red fluorescent protein or yellow fluorescent protein.

5. The method of claim 4, wherein the fluorescent protein is detected by means of a whole-body optical fluorescence imaging system.

6. The method claim 1, wherein the marker gene is detected indirectly.

7. The method of claim 6, further comprising administering to the subject a labeled marker-binding moiety after step (a), wherein the marker gene product of step (a) binds to the labeled marker-binding moiety; and wherein said scanning is after clearance of residual labeled marker-binding moiety not bound to the marker gene product, thereby detecting the labeled marker-binding moiety localized to the tumor and imaging the tumor in the subject.

8. The method of claim 7, further comprising waiting a time-period after administering said labeled marker-binding moiety sufficient to allow at least 67% of unbound labeled marker-binding moiety to clear from the subject.

9. The method of claim 7, further comprising waiting a time-period after administering said labeled marker-binding moiety sufficient to allow at least 80% of

unbound labeled marker-binding moiety to clear from the subject.

10. The method of claim 7, further comprising waiting a time-period after administering said labeled marker-binding moiety sufficient to allow at least 90% of unbound labeled marker-binding moiety to clear from the subject.

11. The method of claim 7, wherein the marker-binding moiety is labeled with a radioisotope suitable for imaging by positron emission tomography, gamma camera or single-photon emission computed tomography.

12. The method of claim 7, wherein the marker-binding moiety contains a nuclide.

13. The method of claim 12, wherein the nuclide is ^{3}H, ^{11}C, ^{14}C, ^{13}N, ^{18}F, ^{123}I, ^{124}I or ^{131}I.

14. The method of claim 7, wherein the labeled marker-binding moiety is imaged by positron emission tomography.

15. The method of claim 7, wherein the labeled marker-binding moiety is imaged by gamma camera or single-photon emission computed tomography.

16. The method of claim 7, wherein the labeled marker-binding moiety is imaged by magnetic resonance imaging.

17. The method of claim 7, wherein the marker gene product is streptavidin.

18. The method of claim 17, wherein the labeled marker-binding moiety is biotin.

19. The method of claim 7, wherein the marker gene product is a receptor.

20. The method of claim 19, wherein the labeled marker-binding moiety is a ligand which binds to said receptor.

21. The method of claim 7, wherein the labeled marker-binding moiety is an

anti-marker gene product antibody.

22. The method of claim 6, further comprising administering to the subject a labeled marker substrate after step (a), wherein the tumor-targeted bacteria expressing the marker gene product of step (a) metabolizes the labeled marker substrate to produce a labeled marker metabolite; and wherein said scanning is after clearance of residual marker substrate not metabolized by the marker gene product, thereby detecting the labeled marker metabolite localized to the tumor and imaging the tumor in the subject.

23. The method of claim 22, further comprising waiting a time-period after administering said labeled marker substrate sufficient to allow at least 67% of non-specific label derived from residual marker substrate not metabolized by the marker gene product to clear from the subject.

24. The method of claim 22, further comprising waiting a time-period after administering said labeled marker substrate sufficient to allow at least 80% of non-specific label derived from residual marker substrate not metabolized by the marker gene product to clear from the subject.

25. The method of claim 22, further comprising waiting a time-period after administering said labeled marker substrate sufficient to allow at least 90% of non-specific label derived from residual marker substrate not metabolized by the marker gene product to clear from the subject.

26. The method of claim 22, wherein the marker gene is wild-type, mutant or genetically engineered herpes simplex virus-thymidine kinase or varicella zoster virus-thymidine kinase.

27. The method of claim 22, wherein the marker substrate is labeled with a radioisotope suitable for imaging by positron emission tomography, gamma camera or single-photon emission computed tomography.

28. The method of claim 22, wherein the marker substrate and marker metabolite are compounds containing a nuclide.

29. The method of claim 28, wherein the nuclide is ^{3}H, ^{11}C, ^{14}C, ^{13}N, ^{18}F, ^{123}I, ^{124}I

or ^{131}I.

30. The method of claim 22, wherein the labeled marker metabolite is imaged by positron emission tomography.

31. The method of claim 22, wherein the labeled marker metabolite is imaged by gamma camera or single-photon emission computed tomography.

32. The method of claim 22, wherein the labeled marker metabolite is imaged by magnetic resonance imaging.

33. The method of claim 22, wherein the labeled marker substrate comprises a labeled 2'-fluoro-nucleoside analogue.

34. The method of claim 1, wherein the tumor-targeted bacteria are *Escherichia coli*, *Salmonella spp.*, *Shigella spp.*, *Streptococcus spp.*, *Yersinia enterocolitica*, *Listeria monocytogenies*, or *Mycoplasma hominis*.

35. A method of monitoring a tumor during the course of a disease in a subject comprising:

(a) administering to the subject a tumor-targeted microorganism containing a marker gene, wherein the tumor-targeted microorganism targets the tumor cells and/or the tumor environment and the marker gene is expressed in the tumor-targeted microorganism, thereby generating a marker gene product;

(b) scanning the subject to detect the marker gene product, thereby imaging the tumor in the subject; and

(c) repeating steps (a) and (b) as needed during the course of the disease in the subject.

36. A method of imaging a tumor in a subject comprising:

(a) administering to the subject a tumor-targeted bacteria, wherein the tumor-targeted bacteria targets the tumor cells and/or the tumor environment;

(b) administering to the subject a labeled compound that is preferentially incorporated into the tumor-targeted bacteria; and

(c) scanning the subject to detect the labeled compound, thereby detecting the tumor-targeted bacteria and imaging the tumor in the subject.

37. The method according to claim 36, wherein the tumor-targeted bacteria is a mutant having an enhanced preference to incorporate the labeled compound.

38. The method according to claim 37, wherein the bacteria is a mutant at the *asd* locus and the labeled compound is diaminopimelic acid.

39. The method according to claim 36, further comprising waiting a time-period after step (b) sufficient to allow at least 67% of unincorporated labeled compound to clear from the subject.

40. The method according to claim 36, further comprising waiting a time-period after step (b) sufficient to allow at least 80% of unincorporated labeled compound to clear from the subject.

41. The method according to claim 36, further comprising waiting a time-period after step (b) sufficient to allow at least 90% of unincorporated labeled compound to clear from the subject.

42. The method of claim 36, wherein the labeled compound is labeled with a radioisotope suitable for imaging by positron emission tomography, gamma camera or single-photon emission computed tomography.

43. The method of claim 36, wherein the labeled compound contains a nuclide.

44. The method of claim 43, wherein the nuclide is ^{3}H, ^{11}C, ^{14}C, ^{13}N, ^{18}F, ^{123}I, ^{124}I or ^{131}I.

45. A method of monitoring a tumor during the course of a disease in a subject comprising:

(a) administering to the subject a tumor-targeted bacteria, wherein the tumor-targeted bacteria targets the tumor cells and/or the tumor environment;

(b) administering to the subject a labeled compound that is preferentially incorporated into the tumor-targeted bacteria;

(c) scanning the subject to detect the labeled compound, thereby detecting the tumor-targeted bacteria and imaging the tumor in the subject; and

(d) repeating steps (a) through (c) as needed during the course of the

disease in the subject.

46. A method of imaging a tumor in a subject comprising:

(a) administering to the subject a tumor-targeted bacteria, wherein the tumor-targeted bacteria targets the tumor cells and/or the tumor environment;

(b) scanning to image an infection caused by the bacteria, thereby detecting and imaging the tumor in the subject.

47. The method according to claim 46, wherein imaging the infection comprises detecting sequestered polymorphonuclear neutrophils at the site of infection.

48. The method according to claim 47, wherein the polymorphonuclear neutrophils at the site of infection are detected using a labeled antibody that detects an antigen present on the polymorphonuclear neutrophils.

49. The method according to claim 48, wherein the antigen is CD15.

50. The method according to claim 47, wherein the polymorphonuclear neutrophils at the site of infection are detected using a labeled chemotactic peptide analog that binds to a receptor present on the polymorphonuclear neutrophils.

51. A method of monitoring a tumor during the course of a disease in a subject comprising:

(a) administering to the subject a tumor-targeted bacteria, wherein the tumor-targeted bacteria targets the tumor cells and/or the tumor environment;

(b) scanning to image an infection caused by the bacteria, thereby detecting and imaging the tumor in the subject; and

(c) repeating steps (a) and (b) as needed during the course of the disease in the subject.

52. A method of imaging a tumor in a subject comprising:

(a) administering to the subject a tumor-targeted bacteria, wherein the tumor-targeted bacteria targets the tumor cells and/or the tumor;

(b) administering a labeled antibody to the subject, wherein the antibody binds to an antigen present on the surface of the tumor-targeted bacteria; and

(c) scanning the subject to detect the labeled antibody, thereby imaging

the tumor in the subject.

53. The method of claim 72, wherein the antigen present on the surface of the tumor-targeted bacteria is an O-antigen, an H-antigen, or an outer membrane protein.

54. A method of monitoring a tumor during the course of a disease in a subject comprising:

(a) administering to the subject a tumor-targeted bacteria, wherein the tumor-targeted bacteria targets the tumor cells and/or the tumor;

(b) administering a labeled antibody to the subject, wherein the antibody binds to an antigen present on the surface of the tumor-targeted bacteria;

(c) scanning the subject to detect the labeled antibody, thereby imaging the tumor in the subject; and

(d) repeating steps (a) through (c) as needed during the course of the disease in the subject.

55. A method of simultaneously imaging and treating a tumor in a subject comprising:

(a) administering to the subject a tumor-targeted microorganism containing a marker gene, wherein the tumor-targeted microorganism infects the tumor and/or the tumor environment and the marker gene is expressed in the tumor-targeted microorganism, thereby generating a marker gene product;

(b) administering to the subject a tumor-targeted microorganism containing a suicide gene, wherein the tumor-targeted microorganism infects cells of the tumor and/or the tumor environment and the suicide gene is expressed in the tumor-targeted microorganism, thereby generating a suicide gene product;

(c) administering to the subject a suicide gene substrate, wherein the tumor-targeted microorganism expressing the suicide gene product of step (b) metabolizes the suicide gene substrate to produce a cytotoxic metabolite; and

(d) scanning the subject to detect the marker gene product, thereby simultaneously imaging and treating the tumor in the subject.

56. A method for simultaneously: (i) imaging or monitoring; and (ii) reducing the volume of or inhibiting the growth of, a solid tumor comprising

administering an effective amount of a first isolated population of facultative aerobic or facultative anaerobic tumor-targeted bacteria suitable for imaging or monitoring

the solid tumor in combination with a second isolated population of facultative aerobic or facultative anaerobic tumor-targeted bacteria suitable for reducing the volume of or inhibiting the growth of the solid tumor.

57. The method according to claim 56, wherein the first or second isolated population of facultative aerobic or facultative anaerobic tumor-targeted bacteria is an isolated population of super-infective, tumor-targeted bacteria.

58. The method according to claim 57, wherein at least one super-infective, tumor-targeted bacteria population is an attenuated population.

59. The method according to claim 58, wherein the attenuated population induces TNFα expression from about 1 to about 75 percent as compared to a corresponding non-attenuated population.

60. The method according to claim 56, wherein at least one isolated population of facultative aerobic or facultative anaerobic tumor-targeted bacteria is *Escherichia coli*, *Salmonella spp.*, *Shigella spp.*, *Streptococcus spp.*, *Yersinia enterocolitica*, *Listeria monocytogenies*, or *Mycoplasma hominis*.

61. The method according to claim 60, wherein at least one isolated population is *Salmonella spp.*

62. The method according to claim 56, wherein the solid tumor cancer is renal carcinoma, mesoendothelioma, bladder cancer, a germ line tumor, a tumor of the central nervous system, breast cancer, prostate cancer, cervical cancer, uterine cancer, lung cancer, ovarian cancer, testicular cancer, thyroid cancer, astrocytoma, glioma, pancreatic cancer, stomach cancer, liver cancer, colon cancer, or melanoma.

63. A method for simultaneously: (i) imaging or monitoring; and (ii) reducing the volume of or inhibiting the growth of a solid tumor comprising

administering an effective amount of a first single colony clone of an isolated population of facultative aerobic or facultative anaerobic tumor-targeted bacteria, which clone replicates preferentially at a tumor site and is suitable for imaging or monitoring the solid tumor in combination with a second single colony clone of an isolated population of facultative aerobic or facultative anaerobic tumor-targeted bacteria, which

clone replicates preferentially at a tumor site and is suitable for reducing the volume of or inhibiting the growth of the solid tumor.

64. The method according to claim 63, wherein at least one single colony clone of an isolated population of facultative aerobic or facultative anaerobic tumor-targeted bacteria is a single colony clone of an isolated population of super-infective, tumor-targeted bacteria.

65. The method according to claim 64, wherein the super-infective, tumor-targeted single colony clone is an attenuated single colony clone.

66. The method according to claim 65, wherein the attenuated single colony clone induces TNFα expression from about 1 to about 75 percent as compared to a corresponding non-attenuated single colony clone.

67. The method according to claim 66, wherein at least one single colony clone of an isolated population of facultative aerobic or facultative anaerobic tumor-targeted bacteria is selected from the group consisting of *Escherichia coli*, *Salmonella spp.*, *Shigella spp.*, *Streptococcus spp.*, *Yersinia enterocolitica*, *Listeria monocytogenies*, and *Mycoplasma hominis*.

68. The method according to claim 67, wherein the bacteria are *Salmonella spp.*

69. A composition comprising a population of tumor-targeted bacteria containing a recombinant streptavidin gene operably linked to a promoter.

70. The composition of claim 69, wherein the promoter is preferentially active in a tumor environment.

71. The composition of claim 69, further comprising a pharmaceutically acceptable carrier.

72. A composition comprising a population of tumor-targeted bacteria, wherein the tumor-targeted bacteria are partially mutant for the *asd* locus.

73. The composition of claim 72, wherein the bacteria is *Salmonella spp.* or *E.*

coli.

74. The composition of claim 72, further comprising a pharmaceutically acceptable carrier.

75. A kit comprising in one or more containers (a) a purified population of tumor-targeted bacteria and (b) a detectably labeled molecule.

76. The kit of claim 75, wherein the bacteria contain a marker gene operably linked to a promoter.

77. The kit of claim 76, wherein the labeled molecule is a labeled moiety which binds to the marker gene product.

78. The kit of claim 77, wherein the labeled molecule is labeled biotin and the marker gene is streptavidin.

79. The kit of claim 77, wherein the labeled molecule is a labeled antibody.

80. The kit of claim 76, wherein the labeled molecule is a labeled substrate of the marker gene product.

81. The kit of claim 75, wherein the labeled molecule is a labeled compound which is preferentially incorporated into the tumor-targeted bacteria.

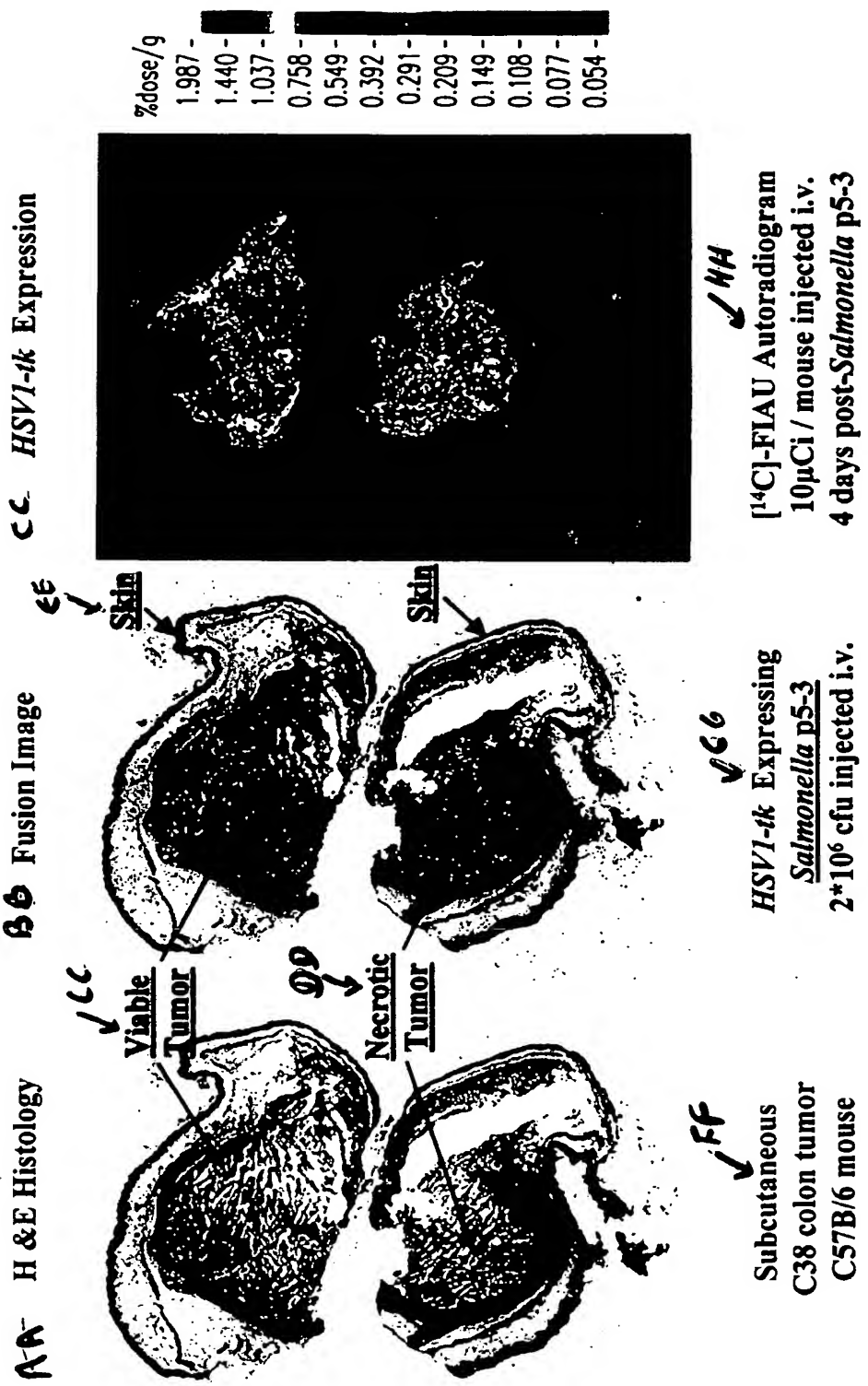
82. The kit of claim 81, wherein the tumor-targeted bacteria is a mutant having an enhanced preference to incorporate the labeled compound.

83. The kit of claim 82, wherein the bacteria is mutant at the *asd* locus and the labeled compound is diaminopimelic acid.

84. The kit of claim 75, wherein the labeled molecule is a labeled antibody that detects an antigen present on polymorphonuclear neutrophils, or a labeled chemotactic peptide analog that binds to a receptor present on polymorphonuclear neutrophils.

85. The kit of claim 75, wherein the labeled molecule is a labeled antibody that detects an antigen present on the bacterial surface.

86. The kit of claim 76, wherein the antigen present on the bacterial surface is an O antigen, H antigen, or an outer membrane protein.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1.440
1.037
0.758
0.549
0.392
0.291
0.209
0.149
0.108
0.077
0.054
Skin
Skin
Viable Tumor
Necrotic Tumor
Subcutaneous C38 colon tumor C57B/6 mouse
HSV1-tk Expressing Salmonella p5-3
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[14C]-FIAU Autoradiogram
10μCi / mouse injected i.v.
4 days post-Salmonella p5-3

FIG. 1

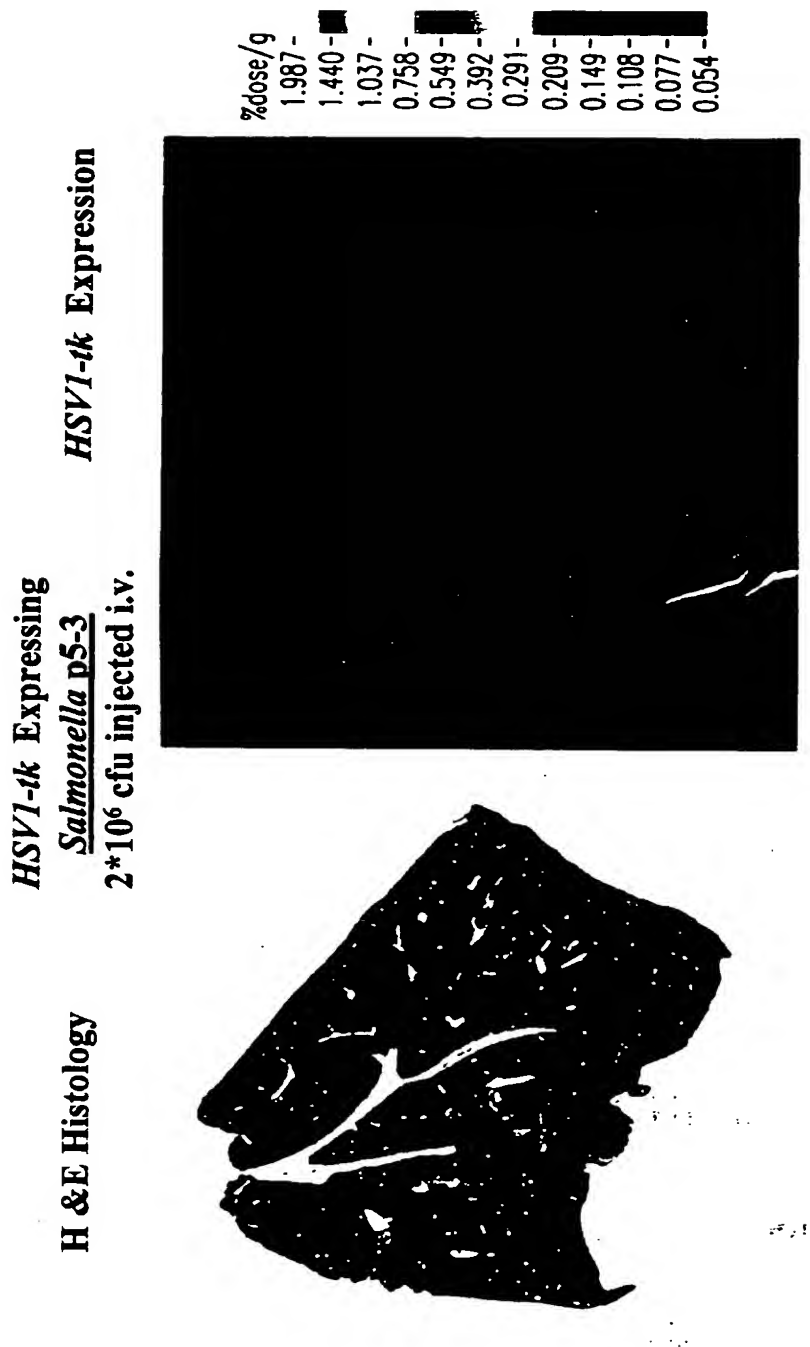
%dose/g
1.987
1.440
1.037
0.758
0.549
0.392
0.291
0.209
0.149
0.108
0.077
0.054
HSV1-tk Expressing
Salmonella p5-3
2*10⁶ cfu injected i.v.
HSV1-tk Expression
[¹⁴C]-FIAU Autoradiogram
10µCi / mouse injected i.v.
4 days post-Salmonella p5-3
H &E Histology
Liver
C57B/6 mouse

FIG.2

FIG.3A

FIG.3B

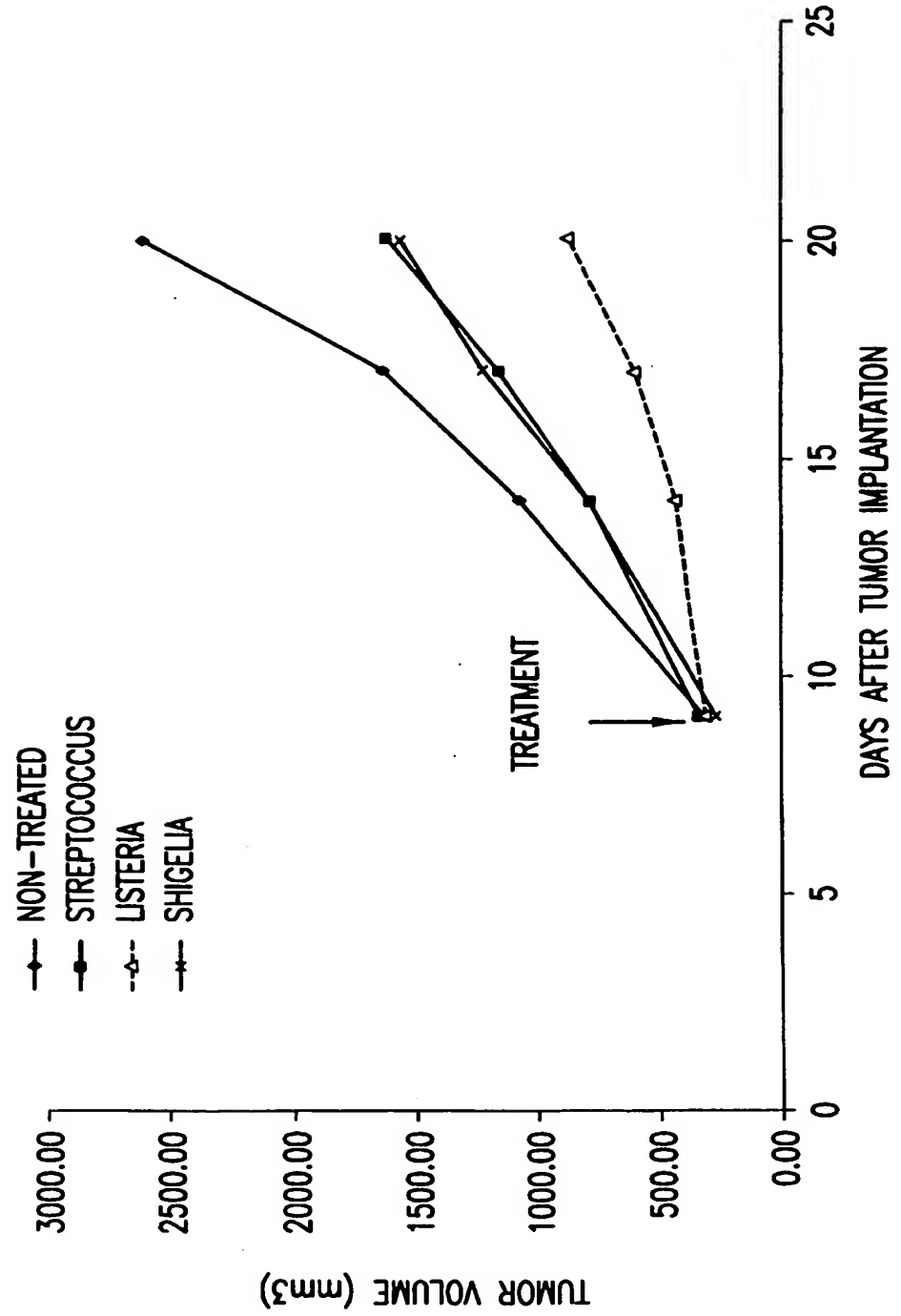
NON-TREATED
STREPTOCOCCUS
LISTERIA
SHIGELLA
TREATMENT
TUMOR VOLUME (mm3)
3000.00
2500.00
2000.00
1500.00
1000.00
500.00
0.00
0
5
10
15
20
25
DAYS AFTER TUMOR IMPLANTATION

FIG.4

SEQUENCE LISTING

<110> VION PHARMACEUTICALS, INC.
SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH

<120> NON-INVASIVE TUMOR IMAGING BY TUMOR-TARGETED BACTERIA

<130> 8002-063-228

<150> 60/157,620
<151> 1999-10-04

<160> 14

<170> FastSEQ for Windows Version 3.0

<210> 1
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> forward primer

<400> 1
gatcccatgg cttcgtaccc cggcc 25

<210> 2
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> reverse primer

<400> 2
ctagaagctt cagtggctat ggcagggc 28

<210> 3
<211> 58
<212> DNA
<213> Artificial Sequence

<220>
<223> oligonucleotide

<221> modified_base
<222> (35)...(43)
<223> n=a, c, g, or t

<400> 3
tgggagctca catgccccgc ccccggccct caccnnnnnn nnngaccgcc atcccatc 58

<210> 4
<211> 51
<212> DNA
<213> Artificial Sequence

<220>
<223> oligonucleotide

<221> modified_base
<222> (27)...(35)
<223> n=a, c, g, or t

<400> 4
ataaggtacc gcgcggccgg gtagcannnn nnnnnggcga tgggatggcg g 51

<210> 5
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 5
tgggagctca catgccccgc c 21

<210> 6
<211> 11
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 6
atgaggtacc g 11

<210> 7
<211> 15
<212> PRT
<213> HSV1

<400> 7
Leu Ile Phe Asp Arg His Pro Ile Ala Ala Leu Leu Cys Tyr Pro
1 5 10 15

<210> 8
<211> 15
<212> PRT
<213> HSV1

<400> 8
Leu Leu Leu Asp Arg His Pro Ile Ala Cys Met Leu Cys Tyr Pro
1 5 10 15

<210> 9
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> forward primer

<400> 9
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<210> 10
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> reverse primer

<400> 10
cggcgatcgg atggcggtcg aggaggaggg 30

<210> 11
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> forward primer

<400> 11
ccatccgatc gccgtcatgc tgtgc 25

<210> 12
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> reverse

<400> 12
ctagaagctt cagtggctat ggcagggc 28

<210> 13
<211> 49
<212> DNA
<213> Artificial Sequence

<220>
<223> forward primer

<400> 13
gtgtgcggcc gcaatattac tgaaatgagc tgttgacaat taatcatcc 49

<210> 14
<211> 39
<212> DNA
<213> Artificial Sequence

<220>
<223> reverse primer

<400> 14
gtgtgatatc gcatgcctgc aggtcgactc tagaattac 39